THE CHEMISTRY, IMMUNOCHEMISTRY AND BIOSYNTHESIS OF BACTERIAL CELL SURFACES, WITH SPECIAL REFERENCE TO ANAEROBIC BACTERIA

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To Carol, Timothy and Christopher

CONTENTS

DECLARATION

I declare that this thesis is my own composition. With the exception of papers 1-4 which describe work carried out as part of my PhD studies at Edinburgh, no part of this thesis has been submitted for a higher degree. In papers 30-33, 37, 41, 43, 44, 46, 47, 53 and 54 I was a major collaborator. In all others I was the senior author and/or the originator of the work described.

Ian R Poxton, June 1991.

ABSTRACT

This thesis records my involvement in bacterial cell surface research which began in 1971 during my PhD programme with early studies on the biosynthesis and chemistry of klebsiella polysaccharides. A detailed study of the chemistry of the pneumococcal teichoic acid followed and here I was introduced to immunochemistry. The structure and immunochemistry of the surfaces of anaerobic bacteria, particularly of the *Bacteroides* species and the clostridia, then become a major interest. *Clostridium difficile* played an important part in this section, where the most significant contribution showed conclusively that it was an infectious agent. Links with the Moredun Research Institute have resulted in collaborative studies on the important veterinary pathogens *Pasteurella haemolytica* and *P. multocida*, and a study of the bacteriology of periodontal disease in sheep. Concurrent with this have been other collaborative studies on bacterial surfaces, in particular the group B streptococci. The major current interest is on the chemistry and immunochemistry of bacterial lipopolysaccharide (LPS) with a particular interest in its interaction with antibodies (leading ultimatly to immunotherapy of septic shock and Gram-negative sepsis) and the environmental influence on LPS expression by bacteria and its subsequent detection.

ACKNOWLEDGEMENTS

In any branch of modern science, teamwork and collaboration are essential. This thesis is the result of such cooperation. My first thanks must go to Ian Sutherland, my PhD supervisor, who was instrumental in much that followed. Jim Lomax was also an important help during these early years in propagating my interests in carbohydrate chemistry and "techniques". It was during my post-doctoral research in Newcastle that the long-term aims were to be established and Sir James Baddiley, Ron Archibald, Ted Tarelli, Peter Lambert, John Coley and David Powell were all important influences. Special thanks to Ted for all his help with ^{13}C-NMR and methylation analysis.

In the past 14 years in Edinburgh many friends and colleagues must be acknowledged. Special thanks go to Professor Gerry Collee who introduced me to anaerobes and has been a constant source of encouragement ever since, and Robert Brown who has been responsible for so much of the technical work, but most especially for running such a good laboratory. All my graduate students and research assistants must be thanked: Madeleine Ip, Chris Cumming, Gary Cousland, Willie Donachie, Marie Byrne, the late Jacquie Sharp, David Smith, Jane McCourtie, Zaiyanu Abdullahi, Thula Wijewardana, Alastair Sutherland, Frances McLouglin and David Nelson all feature as co-authors on one or more publications and their contributions were essential. At Moredun the collaboration with Neil Gilmour, Willie Donachie and their Pasteurella group has

always been fruitful, and John Spence's influence in setting up the broken mouth work is gratefully acknowledged.

Dr Brian McClelland, Director of the SE Scotland Blood Transfusion Service, was responsible for re-awakening my current main interest on bacterial lipopolysaccharide and immunotherapy of endotoxic shock and I cannot thank him enough. The subsequent collaboration with Robin Barclay and colleagues in the Blood Transfusion Service has been, and is still proving a most interesting and worthwhile field of study.

Lastly but by no means least all the junior technical staff and honours students doing project work who have passed through the lab over the years have all had a part to play and must be thanked accordingly.

INTRODUCTION

My first interest in bacterial cell surfaces began in the summer of 1970 when I was given the opportunity, during the vacation prior to my honours year, of working in the Microbiology Department, University of Edinburgh with Dr Ian W Sutherland. The project involved an investigation of the biosynthesis of the exopolysaccharide of Klebsiella aerogenes by following the incorporation of C-14 labelled sugar nucleotide precursors via a lipid intermediate into polysaccharide.

A year later I was awarded a PhD studentship by the Science Research Council to continue the project under the supervision of Ian Sutherland. A thesis entitled 'Carrier lipids involved in the biosynthesis of polysaccharides in Klebsiella aerogenes' was submitted in 1974 and the degree of PhD awarded in 1975. During the course of my PhD programme the techniques were concerned primarily with the biochemistry of the biosynthetic reactions. There was however a necessity to do a certain amount of preparative chemistry in order to produce the enzymes and substrates involved in the reactions. Much of this was done with the help and guidance of Jim Lomax. I also became interested in the chemical analysis of the various polysaccharides synthesised by the mutants that were isolated (1-4).

At the end of my PhD in 1974 I was fortunate to be able to continue and extend my interests in the structural chemistry

of bacterial cell surface carbohydrates by being appointed as a Research Associate on a Medical Research Council grant under the direction of Professor Sir James Baddiley in the Microbiological Chemistry Research Laboratory, Chemistry Department, University of Newcastle upon Tyne. The project was to determine the structure of the C-teichoic acid of Streptococcus pneumoniae. During my three years in Newcastle I began for the first time to become aware of the fascination and the complexity of the chemistry of the bacterial cell surface. Under the expert guidance of Sir James and Ron Archibald I began to get to grips with the intricacies of the chemistry of the pneumococcal teichoic acid, a molecule of great biological interest which offered a challenge to attempt to unravel its structure. With the arrival of Ted Tarelli in Newcastle I was able to use such modern analytical techniques as ^{13}C nuclear magnetic resonance spectroscopy and methylation analysis by combined gas chromatography/mass spectrometry to help elucidate the complex structure which was defying solution by more classical chemical methods (5).

As well as learning chemistry in Newcastle I was able to follow my interests in biosynthesis and, with the aid of two MSc students, David Leak and Mavis Thomas, make some inroads into the biosynthetic incorporation of choline into the cell wall of the pneumococcus (6, 7). It was also in Newcastle that my first interests in immunochemistry began. The teichoic acids of the pneumococcus are important antigens and it seemed appropriate that immunological techniques should be

exploited for their study. Antibodies were raised in rabbits and simple precipitation-in-gel and inhibition of complement induced haemolysis techniques were used.

In 1977 I was appointed, by Professor B P Marmion, to a Lectureship in Bacteriology in the University of Edinburgh Medical School and given space in the Microbial Pathogenicity Research Laboratory which was headed by Professor J G Collee. At this time the work that I had begun on pneumococcal cell wall carbohydrates was terminated as my new department did not have the necessary facilities for me to progress further. I was therefore looking for new areas of research to follow where I could apply the techniques that I had learnt during the preceding six years. The area of the Bacteriology Department in which I found myself had an international reputation as a laboratory in which the relatively new discipline of anaerobic bacteriology was being developed. This is the field that was to become my major area of research for the next 10 or so years.

Anaerobic bacteriology, in 1977, was at the stage of development where it was well recognised that the anaerobic bacteria, especially the non-sporing, Gram-negative rods, were a major cause of abdominal sepsis. The organisms could be isolated, cultured in the laboratory and identified to genus and usually species level by conventional bacteriological techniques. Very little however was known of their structure or how they interacted with the host during

the pathogenic process. These were the areas that I decided to concentrate on initially.

Organisms belonging to the genus Bacteroides were the first group of anaerobes to be investigated, especially the B. fragilis group which comprises the major pathogens of the genus. Both cell surface chemistry and immunochemistry have been investigated in detail. Powerful, modern immunochemical methods such as enzyme-linked immunosorbent assay (ELISA) and immunoblotting were exploited (8-11). Robert Brown our chief MLSO, and Madeleine Ip and Gary Cousland, two postgraduate students, made significant contributions to these studies.

In the late 1970s it became apparent that the best known group of anaerobes, the Gram-positive, spore-forming rods, - the clostridia, still played a major role in serious infections and were in fact responsible for some 'new' infections. A study in which I was ably assisted by Marie Byrne resulted in several publications dealing with the antigenic make-up of clostridia (18-19), in particular Clostridium botulinum and related organisms (20) and C.difficile the organism recently identified as being the cause of antibiotic-associated colitis. The work on C. difficile included some fundamental studies on the microbiology, structure and immunochemistry of the organism (18, 21-26), and a consequence of this was the development of a fingerprinting method (27-28) which could be used for the debated epidemiology of C. difficile infections. This

technique has been applied to a number of collaborative investigations some of which have been published (27, 29-30). We have concluded that C. difficile is an infectious agent that can be transmitted from person to person. A detailed study of the culture, epidemiology and virulence factors of C. difficile has been completed, with Jacquie Sharp submitting a PhD thesis of her contributions to the work.

Several reviews and contributions to books have resulted from my interest in anaerobes (31-36).

Anaerobes have been by no means my only interest in Edinburgh. The interests in cell surfaces of Gram-positive cocci which began in Newcastle have continued and I have been involved in several collaborative projects on streptococci. The most significant was on the carbohydrate antigens of Group B streptococcus where I supervised Chris Cumming on this aspect of his PhD thesis (37-40). A small part was also played in collaboration with Jeremy Bagg and Donald Weir in the investigation of the lectin-mediated adherence mechanisms of some other Gram-positive bacteria (41,42).

Links with the Moredun Research Institute began shortly after my return to Edinburgh and engendered an interest in veterinary microbiology. This has been predominantly on Pasteurella haemolytica and P. multocida cell surface immunochemistry and vaccine development with Neil Gilmour, Willie Donachie and colleagues (43-48). More recently this

collaboration was extended to an intensive study of the microbiology of periodontal disease in sheep (broken mouth) with Jane McCourtie and John Spence, combining my anaerobic and veterinary microbiological interests (49-51).

In 1985, a major collaboration with Robin Barclay and colleagues in the Edinburgh and SE Scotland Blood Transfusion Service was begun. The primary aim of the project was to develop antibodies, either from blood donors or monoclonal, which could be used in the prevention and or treatment of Gram-negative sepsis and endotoxaemia. At the time of writing this thesis the project is extremely active and much of the work is unpublished (because of commercial sponsors). However, several papers either directly or indirectly related to the main thrust of the project have been published (52-56).

The major linking theme throughout all of my work has been an interest in the structure and function of the bacterial cell surface in relation to its role in interactions with the host. Of especial interest has been the 'techniques' side of the work: immunochemical and biochemical methods. This interest has led to several invited chapters and reviews (35, 36, 57, 58) and the production of a book (59) entitled 'Bacterial Cell Surface Techniques', co-authored with Ian Hancock of the University of Newcastle. This is a practical manual for the preparation and analysis of bacterial cell surface components. A final publication worthy of note is a

chapter, co-authored with John Arbuthnott of the University of Nottingham, entitled 'Determinants of Bacterial Virulence' for the latest edition of Topley and Wilson's Principles of Bacteriology, Virology and Immunity (60).

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BUTANOL-SOLUBLE GLYCOSYL TRANSFERASES IN *KLEBSIELLA AEROGENES*

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1. Introduction

The bacterial cell membrane is the site of synthesis of several polymers which are composed, at least in part, of oligosaccharide repeating units. In capsulate Gram negative bacteria such as *Klebsiella aerogenes* these products are mucopeptide, lipopolysaccharide and exopolysaccharide (capsule and slime). In biosynthesis of each such polymer, formation of the oligosaccharides involves transfer of monosaccharides from nucleoside diphosphate sugars to a carrier lipid identified as a phosphorylated C_{55}-isoprenoid alcohol [1–3]. Exopolysaccharide synthesis in *K. aerogenes* follows this pattern [3], the first two reactions in the biosynthesis of the capsule of type 8 strains being [4]:

Lipid–P + UDP–glucose → Lipid–P–P–glucose + UMP
Lipid–P–P–glucose + UDP–galactose → Lipid–P–P–glucose–galactose + UDP

The enzymes responsible for these reactions form part of the membrane complex and earlier attempts to obtain soluble transferases were unsuccessful [4]. They are probably highly hydrophobic molecules resistant to extraction with aqueous solvents except under drastic conditions under which enzyme activity is destroyed.

A possible technique for the extraction and purification of hydrophobic proteins from bacterial cell membranes was provided by the acid–butanol method of Strominger and others [5]. Use of this extraction procedure on cell membranes from *Staphylococcus aureus* solubilised a number of proteins from which an isoprenoid alcohol kinase was purified by low temperature precipitation from butanol followed by chromatography on DEAE-cellulose [6]. Preliminary experiments in our laboratory with *K. aerogenes* indicated that 4–5% of the membrane protein could be extracted with acid butanol (H.A. McArthur, unpublished results). We now report the extraction of two glycosyl transferases from *K. aerogenes* type 8 and the reconstruction of an active enzyme system.

2. Materials and methods

The bacterial strain used was a non-mucoid derivative of *K. aerogenes* A4 (type 8) known to be active in glucose transfer to lipid and in galactose transferases I and II [7]. It was used in preference to the wild type cells when extracellular polysaccharide was absent. It was grown overnight in 30 ℓ of trypticase soy broth (Baltimore Biologicals Laboratories, Baltimore, Md., USA) containing 1% (w/v) glucose in 15 ℓ-fermenters (L.H. Engineering Ltd., Stoke Poges, England), harvested by centrifugation at 10 000 *g* for 20 min and washed in saline (pH 7.5). The bacteria were broken by passage through a French pressure cell (Aminco-American Instrument Co., Inc., Silver Spring, Md., USA) suspended in Tris buffer (600 ml, pH 7.5) and nucleic acids were destroyed by the addition of RNAase and DNAase and gentle shaking for 30 min at 30°C. 'Cell membrane' material was recovered by centrifugation at 50 000 *g* for 1 hr and extracted with acid butanol as described [6].

Ficaprenol for use as glycosyl acceptor was prepared from the leaves of *Ficus elasticus* [8]. Reverse-phase chromatography on silica gel revealed a mixture of C_{50}-, C_{55}- and C_{60}-isoprenoid alcohols. Crude isoprenoid alcohol kinase was prepared from a culture of *Staphylococcus* [6].

Incubation mixtures contained: 100 μg ficaprenol, 0.6% Triton X-100, 0.1 mM dimethyl sulphoxide,

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10 mM $CaCl_2$, 10 mM $MgSO_4$, 50 mM Tris buffer (pH 8.0), 5 mM ATP and UDP–[^{14}C]glucose (50 nmoles, 0.2 μCi) in a total volume of 450 μl. They were prepared by adding the prenol dissolved in light petroleum to each tube and evaporating the solvent under reduced pressure. This was repeated after addition of the butanol solution of protein. Triton, dimethylsulphoxide and buffer were then added and thoroughly mixed with a fine rod. All the other components except the nucleoside diphosphate sugars were added and thoroughly mixed on a gyratory mixer. Immediately after the addition of the UDP–glucose, and at intervals thereafter, samples (50 μl) were withdrawn and pipetted into 4 ml of chloroform/methanol (2:1, v/v). Extraction of lipid-bound monosaccharide and scintillation counting were performed as described previously except that Whatman 1PS papers were used to separate the organic phase from aqueous material. To detect galactose transferase activity, radioactive glucose was omitted from the incubation mixtures and UDP–[^{14}]galactose (50 nmoles, 0.2 μCi) was added.

Table 1
Transfer of [^{14}C]glucose from UDP–[^{14}C]glucose to lipid.

Incubation mixture		Additions	(pmoles glucose transferred/ 30 min)
Complete		–	429
	–ATP	–	0
	–Ficaprenol	–	0
Complete		*Staphylococcus* BSE*	1363
Complete	–ATP	CTP	87
	–ATP	GTP	36
	–ATP	TTP	43
	–ATP	UTP	21

BSE* – butanol soluble enzyme.

The incubation mixtures were prepared as described in the Methods and incubated for 30 min at room temp. after addition of the nucleoside triphosphate. Thereafter the UDP–glucose was added and samples extracted with chloroform/methanol 0, 15 and 30 min after incubation at room temp. (18°C). Each reaction mixture contained 4 mg butanol soluble protein.

3. Results and discussion

3.1. *Protein extraction*

The initial extraction of the bacterial membrane material yielded 4.1–4.8% of the membrane protein in several experiments. Much of this protein precipitated along with other material when the soluble extract was held at 0°C for 36 hr. It was removed by centrifugation. Cooling of the resultant supernatant fluid to –20°C for 16–24 hr yielded a small amount of brownish precipitate which was recovered by rapid centrifugation at –20°C and 8000 *g*. The yield was approx. 0.4% of the membrane protein in the best preparation. Lower yields were obtained when cell breakage was sub-optimal, suggesting that the acid butanol solution only extracts significant amounts of protein from bacterial membranes and not from whole bacteria. The coloured precipitates were redissolved in butanol and kept at 2–4°C. A small amount of material precipitated from the butanol solution after 1–2 weeks at this temperature. The mixture was normally homogenised gently before use.

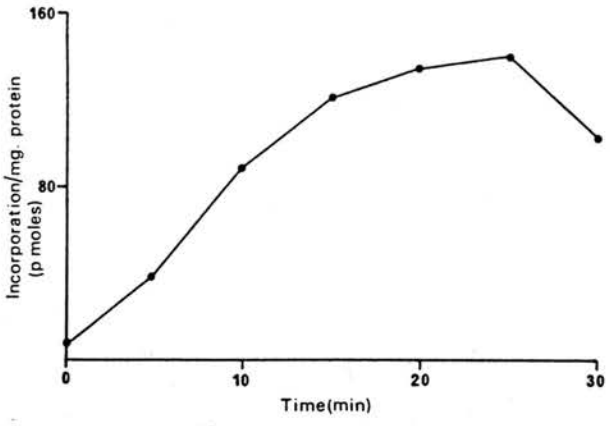

Fig. 1. Transfer of [^{14}C]glucose to lipid by butanol soluble enzyme. Ordinate: Incorporation/mg protein (pmoles). Abscissa: Time (min). Incubation mixtures prepared as described were held for 30 min at room temp. after addition of ATP. UDP-glucose was added and samples (50 μl) withdrawn at 5 min intervals to measure incorporation into chloroform extractable material.

3.2. *Enzymatic activity*

The ability of the bacterial extracts to transfer glucose and galactose from the UDP-monosaccharides to lipid was tested at all stages of the preparation. The initial activity of the cell lysates from the pressure cell was confirmed for both glucose and galactose transfer. Activity was also found in the butanol extracts even though organic solvent was present. On the basis of

activity per mg protein, there was a considerable loss of enzyme function at this stage, transfer being approx. 55–60% less than in the original cell lysates. This may, in part, be due to the presence of butanol in the aqueous incubation mixtures. The procedures used, in which some protein is precipitated and the active protein can only with difficulty be resuspended in aqueous solutions, also prevent accurate comparisons.

The expected reaction is the transfer of glucose-1-phosphate to prenol-phosphate. Transfer to free prenol was not expected and no glucose was found in the organic phase following incubation of UDP–glucose with ficaprenol and the butanol soluble protein. The addition of ATP permitted transfer of glucose to chloroform-soluble material. Consequently, incubation mixtures were kept for 30 min at room temp. to allow phosphorylation of the lipid prior to addition of the UDP–monosaccharides. Although other nucleoside triphosphates could replace ATP, none were as effective as judged by subsequent glucosyl transfer (table 1). These observations indicate that the butanol extracts contain an isoprenoid alcohol kinase resembling that reported by Higashi et al. [5]. The addition of a crude preparation of this enzyme from *Staphylococcus* [6] stimulated the incorporation of glucose but was unable to replace the *Klebsiella* preparation as a source of glucosyl transferase.

Incorporation of glucose from the nucleoside diphosphate monosaccharide into lipid increased with time to a maximum after 25 min (fig. 1). Thereafter a decrease occurred. The reason for this is not known although similar results have been obtained with membrane preparations prior to butanol extraction. The glucosylated lipid may be unstable under the experimental conditions, or other enzymes present in the butanol extract may affect this product. Galactose incorporation resembled that of glucose and in a typical experiment, replicate tubes in which labelled substrates were UDP–[^{14}C]glucose and UDP–[^{14}C]galactose respectively, incorporated 107 pmoles of glucose and 87 pmoles of galactose after 10 min. We have not yet obtained the ratio glucose:galactose 1:2 found in membrane preparations of type *8* strains [4, 7]. The galactosyl transferase II [7] may therefore be absent from the soluble proteins. The reproducibility of hexose incorporation has varied considerably between experiments and this can almost certainly be ascribed to failure to achieve uniform suspension of the hydrophobic protein and lipid.

Recovery of glucosylated lipid from the incubation mixtures by the standard chloroform methanol extraction procedure was followed by chromatography on paper and on silica gel (TLC). On paper, after descending chromatography in ethanol/1 M ammonium acetate, pH 7.3 (7:3, v/v) 1 cm strips of the paper were counted in scintillation fluid. Most of the radioactivity moved with $R_{galactose}$ 1.21 but a small amount was also detected with R_{gal} 0.4 and 0.1. The major peak corresponds well to the position of glucosylated lipid from normal incubation mixtures with membrane material [4]. On TLC in diisobutyl ketone/acetic acid/water (20:15:2) most of the radioactivity had R_f 0.2 with a smaller amount having R_f 0.47. Preparations of mannosyl lipid showed a similar mobility to the major product [9].

Further attempts to separate and purify the glycosyl transferases from *K. aerogenes*, which are involved in the synthesis of the exopolysaccharides of this genus, are continuing. It is of interest that in earlier studies, using a different strain of *K. aerogenes*, extraction of membranes with *neutral* butanol at –25°C left the glycosyl transferases of this strain in the residual particulate material [3].

Acknowledgements

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Isoprenoid Alcohol Kinase – a Third Butanol-soluble Enzyme in *Klebsiella aerogenes* Membranes

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Isoprenoid alcohols function in bacteria as the acceptors on which polysaccharide molecules are assembled. In this role they are involved in the biosynthesis of mucopeptide (Anderson, Matsuhashi, Haskin & Strominger, 1965), lipopolysaccharide (Wright, Dankert & Robbins, 1965), teichoic acids (Anderson, Hussey & Baddiley, 1972) and extracellular capsular or slime polysaccharides (Sutherland & Norval, 1970; Troy, Frerman & Heath, 1971). In growing *Klebsiella aerogenes* three of these polymers are formed, but only the extracellular polysaccharide continues to be synthesized after the bacteria have reached the stationary phase of growth. This indicates that there must be some kind of control to ensure that the isoprenoid alcohol of the organism is available for manufacture of the essential wall polymers during growth but can also function in capsule formation during and after active growth. There is some indication that conditional mutants presumed to be defective in isoprenoid lipid content synthesize only the wall polymers until growth has ceased, when capsular polysaccharide synthesis commences (Sutherland, 1972).

A possible control mechanism for isoprenoid alcohol in *Staphylococcus aureus* was suggested by Strominger and his colleagues following the discovery of an isoprenoid alcohol kinase and a C_{55}-isoprenoid alcohol phosphatase in that species (Sandermann & Strominger, 1972; Willoughby, Higashi & Strominger, 1972). The two enzymes were thought to control the carrier lipid – the kinase making it available, and the phosphatase removing it from the systems requiring it. The isoprenoid alcohol enters the biosynthetic cycle for mucopeptide or for lipopolysaccharide as the phosphate. Subsequently, it accepts a monosaccharide-1-phosphate moiety from a nucleoside diphosphate sugar. After addition of various other monosaccharides or similar compounds, transfer of the oligosaccharide formed to an appropriate acceptor molecule leaves isoprenoid lipid as a pyrophosphate. It is unable to re-enter the biosynthetic cycle for the polymers until it has been dephosphorylated by the specific phosphatase. A second enzyme accomplished further dephosphorylation to yield the free isoprenoid alcohol. The kinase described by Sandermann & Strominger (1972) rephosphorylated free isoprenoid alcohol to the active monophosphate form. If this control operates, such systems will be widely distributed among bacteria and may have an even more important role in those species which possess extracellular polysaccharides in addition to wall polysaccharides.

In general, membranes can be prepared more readily from Gram-positive than from Gram-negative bacteria to permit extraction of the membrane-bound enzymes by the acid–butanol technique of Sandermann & Strominger (1972). Extraction of whole *Klebsiella aerogenes* with acid butanol yielded little protein and none with detectable enzymic activities. When the *K. aerogenes* were broken in an Aminco pressure cell the membranes (contaminated with wall material) could be separated from unbroken bacteria. Acid butanol extraction then

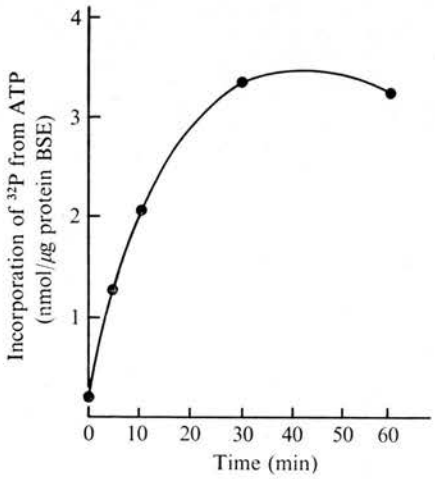

Fig. 1. C_{55} alcohol phosphokinase activity in crude butanol-soluble enzyme (BSE). The activity of crude butanol-soluble extracts was tested by removing 50 μl samples from the incubation mixture and applying them directly to paper, as described by Sandermann & Strominger (1972). After development of the chromatograms for 4 to 5 h, the front area was cut out and its radioactivity estimated directly in a toluene-based scintillation fluid.

dissolved up to 5% of the membrane protein. Two enzymic activities, a glucosyl-phosphate transferase and a galactose transferase, both essential for synthesis of the capsular polysaccharide of *K. aerogenes* type 8, were detected (Lomax, Poxton & Sutherland, 1973). In whole bacteria, the glucose-1-phosphate transferase requires the presence of C_{55} isoprenoid alcohol phosphate as acceptor in the reaction:

Isoprenoid alcohol phosphate + UDP-glucose → Isoprenoid alcohol-P-P-glucose + UMP.

Two non-mucoid mutants of *K. aerogenes* type 8 were used as sources of membrane material, because membranes from capsulate bacteria were recovered with lower yields. After breakage of the bacteria, membranes were recovered by differential centrifugation and extracted three times with acid butanol [butanol–6 M-pyridinium acetate (8:3), pH 4·2, at room temperature]. Partial purification was achieved by precipitation from solvent solution at 0 °C (inactive material) and then at −20 °C (active protein). The brownish precipitate from each preparation was redissolved in butan-1-ol and held at 0 °C. This was used as 'enzyme'. It lacked endogenous acceptor and this was normally replaced with added ficaprenyl phosphate prepared by chemical phosphorylation of ficaprenol (Stone, Wellburn, Hemming & Pennock, 1967). Alternatively, ficaprenol or the C_{55}-isoprenoid alcohol from bacteria (bactoprenol) and ATP could replace the alcohol phosphate, suggesting that the 'enzyme' contained a kinase with similar specificity to that described by Sandermann & Strominger (1972). When 'enzyme' and bactoprenol, which had been dried together under vacuum, were suspended in a buffer containing: 50 mM-tris-HCl pH 8·5, 10 mM-Mg^{++}, 100 mM-P_i, 600 mM-dimethyl sulphoxide, 0·1% 2-mercaptoethanol and 0·3% Triton X-100, addition of 0·05 μCi (0·1 μmol) of ATP-γ^{32}-P yielded a radioactive product soluble in chloroform. This product moved at the solvent front on paper chromatography in isobutyric acid + 1 M-ammonia (5:3). Results from the two mutant strains were identical. Assay of lipid phosphate formation by this technique (Sandermann & Strominger, 1972) showed that the reaction increased with time over a period of about 30 min. Thereafter, a slight but reproducible decrease in product occurred (Fig. 1). This may have been due to the presence of a specific phosphatase in the reaction mixture. Similar results were obtained when ficaprenol was used

as acceptor instead of the bacterial C_{55} isoprenoid alcohol. Controls without added acceptor did not transfer phosphate to lipid-soluble material. Paper chromatograms of complete incubation mixtures scanned in a 4 π scanner showed three peaks, two of which were unreacted ATP and inorganic phosphate, while the third, at the solvent front, was assumed to be isoprenoid alcohol phosphate.

To test the identity of the product of enzyme action, incubation mixtures were extracted with chloroform + methanol (2:1, v/v). The chloroform solutions were thoroughly washed with 0·9% (w/v) NaCl and separated from the aqueous phase by passage through Whatman 1PS papers. Thin-layer chromatography of the chloroform solution in diisobutyl-ketone + acetic acid + water (40:25:5, by vol.) revealed a single radioactive product with R_F 0·6. This is similar to the mobility in this solvent of chemically prepared ficaprenyl or bactoprenyl phosphate run on the same chromatograms and stained with a solution of 5% (w/v) *p*-anisaldehyde in a solution of concentrated H_2SO_4 + 90% ethanol (1:18). Some other isoprenoid alcohols of various chain lengths may also act as substrates, although less effectively than ficaprenol or bactoprenol.

The presence of an isoprenoid alcohol kinase, the third butanol-soluble enzyme isolated from *K. aerogenes* membranes, supports the suggestion of Sandermann & Strominger (1972) that enzymic phosphorylation and dephosphorylation of C_{55} isoprenoid alcohols provides a regulation mechanism for the synthesis of polysaccharides by bacterial membranes. If this is the role of the kinase, it should prove to be an enzyme of widespread occurrence. As yet, it has been found only in one Gram-positive and one Gram-negative bacterial species, but preliminary results (K. Jann, H. Kopmann and I. W. Sutherland, unpublished results) suggest that it can also be isolated from *Escherichia coli* by the techniques described here. It is probable that although a common means of enzyme extraction can be used for membranes from Gram-positive and Gram-negative bacteria, there are sufficient differences in the enzymes from different bacteria to require distinct purification procedures.

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Isolation of Rough Mutants of *Klebsiella aerogenes* and their Synthesis of Polysaccharides

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SUMMARY

Two mutants which lacked both capsular and lipopolysaccharide O-antigen polysaccharides were isolated from *Klebsiella aerogenes* serotype 2 by phage selection; these were designated rough mutants. The polysaccharide fractions solubilized by partial acid hydrolysis of the lipopolysaccharide from both the wild type and mutants were chromatographed on Sephadex G-50. Analysis of the fractions obtained confirmed that the rough mutants lacked the galactan portion of the molecule, which is analogous to the Salmonella O-antigen polysaccharide.

Membranes prepared from wild-type *K. aerogenes*, from a non-mucoid strain (lacking capsule only), and from one of the rough mutants were used in incubation mixtures to compare the biosynthesis of polysaccharides by these organisms. The incorporation of sugar nucleotides into both lipid intermediates and polymer was followed. Results show that the transferases were apparently present in all membranes, while the polymerases were absent in both the non-mucoid and rough mutants.

INTRODUCTION

The lipopolysaccharides (LPS) of *K. aerogenes* do not appear to have the same relatively simple basal structure as those of *Salmonella* spp. and *Escherichia coli*. The polysaccharide is a complex mixture of fragments varying in both molecular weight and monosaccharide content (Sutherland & Wilkinson, 1966; Koeltzow, Epley & Conrad, 1968). A branched galactan has been proposed to be analogous to the O-antigen region of the Salmonella LPS, and other fragments of polysaccharide may represent intermediate structures in the biosynthesis of the complete LPS (Koeltzow & Conrad, 1971).

During biosynthetic studies on the capsular polysaccharide of *K. aerogenes*, Sutherland & Norval (1970) found that sugar nucleotide precursors for the capsular polysaccharide could also be incorporated into lipopolysaccharide. If the O-antigen region of the *K. aerogenes* LPS is synthesized in an analogous manner to that in Salmonella (Nikaido, 1973), then it must be assumed that lipid intermediates isolated from polysaccharide-synthesizing cell-free systems are a mixture of both LPS and capsular polysaccharide precursors.

The aim of this study was to isolate mutants from non-capsulate strains, which lacked the O-antigen, and to use such mutants, together with non-capsulate strains, to obtain information on the possible fate of sugar nucleotide precursors in cell-free systems. *Klebsiella aerogenes* serotype 2 was chosen for this work because its capsular polysaccharide contained mannose, but no galactose – the major O-antigen component. The structure

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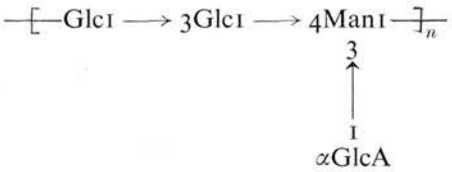
of the *K. aerogenes* serotype 2 capsule has been elucidated by Gahan, Sandford & Conrad (1967):



METHODS

Strains and media. Klebsiella aerogenes serotype 2, NCTC5055, a non-capsulate mutant M4 which had been isolated previously in this laboratory (using a method similar to that described below) from nitrosoguanidine-treated NCTC5055 bacteria by visual examination for non-mucoid colonies, and the two rough mutants (isolated as described below) were grown in 1 l BBL trypticase soy broth in 2 l Erlenmeyer flasks incubated at 30 °C on an orbital shaker.

Bacteriophage F418, a strain from the departmental collection, was isolated from local sewage by the method described by Sutherland & Wilkinson (1965). It was maintained as a high-titre suspension, filter-sterilized, in nutrient broth at 4 °C.

Materials. Sugar nucleotides were purchased from the Boehringer Corp., London, and UDP[^{14}C]glucose and GDP[^{14}C]mannose were obtained from The Radiochemical Centre, Amersham, Buckinghamshire.

Mutagenesis. Portions (100 ml) of exponential-phase cultures of non-mucoid strains were washed once in 0·85 % (w/v) sodium chloride, resuspended in 10 ml 0·1 M-citrate/phosphate buffer, pH 6·1, containing 1 mg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Koch-Light), and left at room temperature for 75 min. The bacteria were then washed twice with 10 ml 0·85 % NaCl, resuspended in a further 10 ml, and 1 ml of the suspension was inoculated into 100 ml trypticase soy broth and incubated for 12 h.

Phage selection. Portions (5 ml) of the 12 h cultures which had undergone mutagenesis were used to inoculate broth (100 ml). After incubation for 4 h, samples were spread on to plates of Oxoid nutrient agar supplemented with 1 % (w/v) glucose. The excess was removed with a sterile pasteur pipette, and the plates were allowed to dry. Drops of phage F418 (4×10^8 plaque-forming units/ml) were spotted on to the plates, the plates were dried, and then incubated at 30 °C overnight. Colonies of phage-resistant mutants were picked from the centre of the zones of clearing and streaked out for single colonies. Colonies which still appeared to be non-mucoid were tested for autoagglutinability by inoculation into 10 ml trypicase soy broth in a test-tube. Those which autoagglutinated (a property of Salmonella rough mutants) after 12 h incubation were grown in 1 l trypticase soy broth for the preparation and analysis of their lipopolysaccharide.

Preparation of lipopolysaccharide. Lipopolysaccharide was extracted from freeze-dried bacteria by the hot aqueous-phenol procedure of Westphal & Lüderitz (1954). The upper aqueous phase was dialysed against running water for 16 h, concentrated to a small volume by rotary evaporation, and centrifuged at 100000 *g* for 4 hr. The lipopolysaccharide, which pelleted as a clear gel, was washed, suspended in distilled water and freeze-dried.

Hydrolyses. (i) Total hydrolysis of the glycosidic bond was achieved by treatment with 1 M-H_2SO_4 at 100 °C for 3 h in a sealed tube. Neutralization was carried out by adding excess Dowex 2 (HCO_3^- form) resin, washing the resin free of hydrolysate in a small no. 1

sintered glass-funnel with distilled water, and finally concentrating the aqueous solution to a small volume by rotary evaporation.

(ii) For partial hydrolysis, 10 to 20 mg LPS was dissolved in 2 ml 1 % (v/v) acetic acid, sealed in a glass tube and hydrolysed for 90 min in a boiling water bath. The coagulated lipid A was removed by centrifuging and the soluble polysaccharide solution was filtered through a no. 4 sintered-glass funnel and freeze-dried.

Paper chromatography. Hydrolysed polysaccharides were analysed by descending chromatography on Whatman no. 1 paper, irrigating with butan-1-ol/pyridine/water (6:4:3, by vol.) for 24 h. Saccharides were detected using the alkaline silver-nitrate reagents of Trevelyan, Procter & Harrison (1950). Pentoses and hexoses were differentiated by spraying with saturated aniline oxalate in water and heating to 105 °C.

Column chromatography. The polysaccharide fraction of partially hydrolysed LPS was chromatographed on Sephadex G-50 (Pharmacia). The eluting buffer was pyridinium acetate (4 ml pyridine and 10 ml acetic acid made up to 1 l with distilled water; Schmidt, Jann & Jann, 1969). Fractions (0·25 ml) were tested with the phenol–sulphuric acid reagents of Dubois *et al.* (1956).

Cell-free incubation mixtures. Cell suspensions (30 %, wet w/v; 5 ml) in 0·85 % NaCl contained in glass tubes surrounded by an ice/ethanol mixture, were disrupted in an M.S.E. 100 W ultrasonic distintegrator set at maximum amplitude for 3×30 s.

Unbroken cells were removed by centrifuging at 5000 ***g*** for 5 min at 4 °C. The membranes were sedimented at 100000 ***g*** for 1 h at 0 °C, suspended in ice-cold distilled water at a concentration of 20 to 30 mg protein ml^{-1} and stored as 1 ml portions at −20 °C.

The incubation mixture, for following the incorporation of sugars from their nucleotide precursors into lipid intermediates and polymer, contained: 200 µl 50 mM-Tris/HCl buffer, pH 8; 25 µl 0·1 M-$MgCl_2$, 10 µl each of unlabelled sugar nucleotides (40 mM); 20 µl ^{14}C-labelled sugar nucleotide (0·1 or 0·25 µCi); and 200 µl membrane suspension (4 to 6 mg protein).

Assay of products from incubation mixtures. Incorporation into lipid intermediates was followed by extracting samples (50 µl) with 4 ml chloroform/methanol (2:1, v/v) at 60 °C for 5 min. This was partitioned with 1 ml 0·85 % NaCl and vortexed. After separating the phases with Whatman 1PS (phase separating) papers, the organic phase was evaporated and its radioactivity was measured in a Packard Tricarb liquid scintillation spectrophotometer, model 3330 (Packard Instruments, Caversham, Berkshire), using 10 ml Triton scintillation fluid (9·1 g PPO, 0·22 g dimethylPOPOP and 500 ml Triton X-100 in 1·16 l toluene).

Incorporation into polymer was followed by spotting samples (5 µl) from the incubation mixtures on to Whatman no. 1 paper and irrigating with ethanol/1 M-ammonium acetate, pH 7·3 (7:3, v/v). The origins were cut out and counted in a toluene scintillation fluid [0·5 % (w/v) PPO in toluene].

Protein was determined by the method of Lowry *et al.* (1951), heptose and 2-keto-3-deoxyoctonic acid (KDO) by the methods of Osborn (1963), and glucosamine by the method of Strominger, Park & Thompson (1959). Glucose was determined using the glucose oxidase reagents (Boehringer) and galactose by the 'Galactostat' galactose oxidase reagent (Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.).

Table 1. *Sugar composition of K. aerogenes LPS (expressed as % dry wt LPS)*

Strain	Yield*	KDO	Heptose	Glucose	Galactose	Glucosamine
NCTC5055 (wild type)	2·5	1·5	5·6	7·5	23·2	5·2
M4	2·3	1·4	5·6	7·0	30·7	6·7
10B	1·3	1·7	7·6	14·2	0·2	7·8
36B	1·2	1·7	6·9	12·5	0·2	10·3

* % dry wt bacteria.

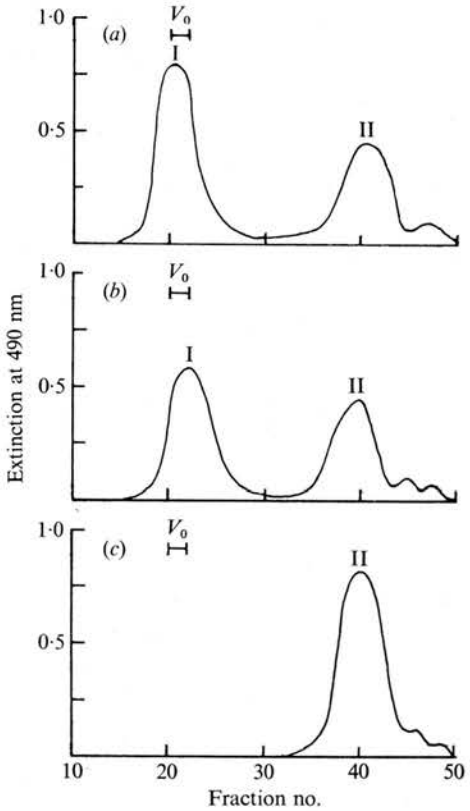


Fig. 1. Elution profiles of polysaccharide fractions obtained after partial hydrolysis of LPS from: (*a*) wild-type (NCTC5055) *K. aerogenes* serotype 2; (*b*) M4 non-mucoid strain; and (*c*) rough mutant 10B. A 250 × 20 mm column of Sephadex G-50 was eluted with pyridinium acetate buffer (described in Methods). Fractions (1 ml) were collected, and 0·25 ml of each was analysed for carbohydrate by the phenol-sulphuric method of Dubois *et al.* (1956). Fractions 18 to 25 (peak I) and 36 to 42 (peak II) were pooled.

RESULTS

Selection and characterization of mutants

After mutagenesis, phage-resistant mutants were selected from the non-mucoid strains of *K. aerogenes*. Mutants designated 10B and 36B were both non-mucoid and autoagglutinable and both could possibly be rough mutants. Other phage-resistant mutants were isolated, but these appeared to be revertants to the capsulated form.

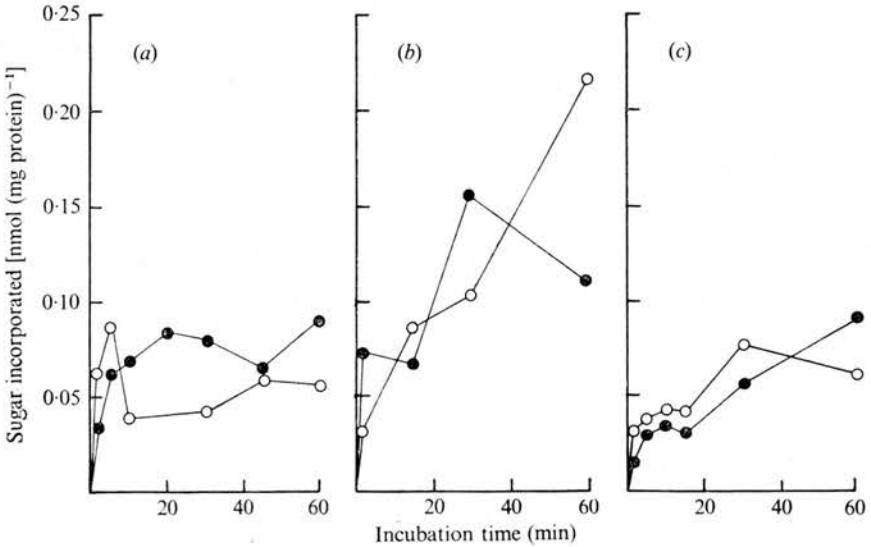


Fig. 2. Incorporation of radioactivity from UDPglucose (●) and GDPmannose (○) into chloroform/methanol soluble material by serotype 2 *K. aerogenes* cell-free systems: (*a*) wild-type (NCTC5055); (*b*) mutant M4; (*c*) mutant 10B. Incubation mixtures were set up as described in Methods. Samples (50 μl) were removed, extracted with chloroform/methanol (2:1, v/v) and the organic phase was assayed for radioactivity.

The LPS was extracted from wild-type bacteria, mutants 10B and 36B, and from a non-mucoid strain (M4) of *K. aerogenes* which has apparently normal LPS. Acid hydrolysis (1 M-H_2SO_4, 3 h, 100 °C) of the intact LPS, followed by paper chromatography, showed that the normal LPS from wild type and M4 was composed of glucose and galactose, with a minor amount of glucosamine. The LPS from mutants 10B and 36B totally lacked galactose. Quantitative analysis (Table 1) suggested that mutants 10B and 36B were of the required type i.e. they apparently lacked the O-antigen (galactan).

Partial acid hydrolysis was carried out on the LPS from wild-type, M4 and 10B strains, and the soluble polysaccharides were fractionated on Sephadex G-50. The elution profiles are shown in Fig. 1. The fractions corresponding to peaks I and II were pooled, reduced in volume and hydrolysed in 1 M-HCl for 3 h at 100 °C. Paper chromatography of the products showed that the peak I fraction was composed almost entirely of galactose; a trace of a pentose which appeared to be xylose, co-chromatographed with it. The peak II fraction appeared to consist entirely of glucose. Gluosamine was absent from both peaks. Quantitative analysis of both 10B and M4 LPS peak II fractions showed that the heptose content of each was approximately 2 % while both preparations contained 1·5 % KDO.

Incorporation of sugars into lipid intermediates

Earlier work (Sutherland & Norval, 1970) showed that membrane preparations from *K. aerogenes* possess a very active UDPgalactose-4-epimerase. This meant that if either UDPglucose or UDPgalactose was added to an incubation mixture they could be interconverted. If the UDPgalactose and UDPglucose were labelled with ^{14}C in the hexose part of the molecule and added to an incubation mixture containing membranes of *K. aerogenes* serotype 2, then it would be expected that the radioactivity would be incorporated into both lipopolysaccharide and exopolysaccharide lipid intermediates. Radioactivity from labelled

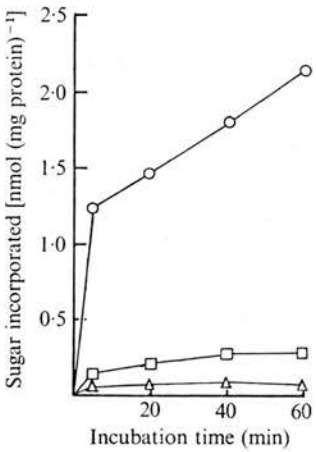


Fig. 3. Incorporation of radioactivity from GDPmannose into polymer. Samples (5 μl) from the same incubation mixtures as in Fig. 2 were spotted on to paper and run in ethanol/1 M-ammonium acetate (7:3, v/v). The origins were assayed for radioactivity, ○, Wild-type (NCTC5055) membranes; □, mutant M4 membranes; △, mutant 10B membranes.

GDPmannose, however, would be expected to be incorporated exclusively into exopolysaccharide lipid intermediates.

Figure 2 compares the incorporation of radioactivity from ^{14}C-labelled UDPglucose and GDPmannose into material soluble in chloroform/methanol (2:1, v/v) by cell-free incubation mixtures prepared from wild-type (NCTC5055), non-mucoid (M4) and rough, i.e. lacking both O-antigen and capsule, (10B) strains of serotype 2 *K. aerogenes*.

Incorporation of mannose into polymer

Much of the radioactivity from UDPglucose is known to be incorporated into glycogen in the cell-free systems described above (unpublished results), and so its incorporation was not followed in this study. Figure 3 shows the incorporation of radioactivity from GDPmannose (in the same incubation mixtures as in Fig. 2) into polymeric material.

DISCUSSION

The O-antigen and LPS structures of Salmonella are now well defined as heteropolysaccharides attached to a defined core structure (Wright & Kanegasaki, 1971). These polymers are the receptors for various bacteriophages, but the receptor sites for the bacteriophages of *K. aerogenes* are not very well understood. Many of these phages can induce capsule degrading enzymes, while others require the capsule as a receptor site (Sutherland, 1972). Phage F418, however, cannot attach to encapsulated bacteria (unpublished observation) and resistance to this phage by non-capsulated bacteria could occur in one of three ways: (i) by reversion to capsulation (mucoid); (ii) by development of immunity through the bacterium becoming lysogenic for that phage; or (iii) by loss or alteration of the phage receptor site through mutation.

In the *K. aerogenes* mutants 10B and 36B, it appears that (iii) has occurred. The galactan (which is analogous to the O-antigen in *Salmonella* spp. or *Escherichia coli*) has been lost. Other phage-resistant mutants appear to be revertants to mucoidness and apparently the capsule protects the receptor sites.

Gel filtration of the polysaccharide fraction obtained from partially hydrolysed normal LPS (from wild-type bacteria or strain M4) resulted in the separation of two fractions. A similar separation into two fractions was obtained with *E. coli* LPS (Schmidt *et al.*, 1969) and with *Pseudomonas aeruginosa* LPS (Fensom & Meadow, 1970). The larger molecular weight fraction, excluded from the gel, represents the O-antigen (galactan). The material included on the column, by analogy with the other bacteria studied, probably represents core polysaccharide. This core is not of the Salmonella type – it does not contain galactose. We found no evidence for a heterogeneous LPS as reported by Koeltzow & Conrad (1971) for NCTC243, serotype 2 *K.* (*Aerobacter*) *aerogenes*. Nimmich & Korten (1970) studied the chemical composition of lipopolysaccharides from 12 different O serotypes of Klebsiella. Several strains had a high proportion of galactose in their LPS whilst others almost entirely lacked this sugar. In these galactose-deficient strains, mannose or rhamnose apparently replaced the galactose. It is possible that all Klebsiella O-antigens are homopolysaccharides.

When the incorporation of radioactivity from the sugar nucleotides into lipid intermediates was followed (Fig. 2), it was seen that both mannose and glucose (or galactose) were incorporated regardless of the sources of the membrane. The wild-type (NTCC5055) cell-free system showed an initial high rate of incorporation of radioactivity from GPDmannose followed by a decrease, then a steady state; this was typical of the formation of a lipid intermediate which was turning over into polymer [see, for example, Anderson *et al.* (1965) for peptidoglycan lipid intermediates; Nikaido, Nikaido & Nakae (1971) for LPS O-antigen formation; or Schultz & Elbein (1974) for mannan synthesis in *Mycobacterium smegmatis*]. The initial peak was not so apparent in the incorporation of GDPmannose by M4 and 10B membrane preparations, but these both showed a definite formation of lipid intermediates.

The observed incorporation of radioactivity from both UDPglucose and GDPmannose into lipid by M4 cell-free preparations could be explained if lipid (i.e. undecaprenyl phosphate) were present in 'normal' amounts and the enzymes responsible for the addition of sugars from their nucleotide sugar precursor to the lipid (transferases) were still present, but turnover into polymer was somehow prevented. This might result in the accumulation of sugars on the lipid. In the case of mutant 10B, lipid would presumably be required only for peptidoglycan synthesis and might be at a lower than normal level. The transferases, however, still appear to be present. The results suggested that the defects in these mutants might be at the level of assembling the oligosaccharide units, which have been assembled on the lipid, into completed molecules outside the membrane. The increased incorporation of sugars on to lipid, as observed for M4 membranes, is characteristic of many of the non-mucoid mutants of *K. aerogenes* (Norval & Sutherland, unpublished results). An accurate assessment of the lipid intermediates which were being formed is still not possible, because of the very low levels of sugars which are assembled on to the lipid. So far no system has been developed for *K. aerogenes* in which the different lipid intermediates can be separated. Attempts with concanavalin A have proved unsuccessful.

It should be noted that the incorporation of radioactivity from GDPmannose into lipid in Fig. 2, especially by M4 membranes, was dependent on the presence of UDPglucose in the incubation mixture and there is no evidence for a polymannosyl-lipid or any mannose-containing polymer other than the expopolysaccharide in *K. aerogenes*.

Confirmation that no mannose-containing polymer (exopolysaccharide) was being synthesized by the cell-free system from either mutant is provided in Fig. 3. As mentioned above, the incorporation of radioactivity from UDPglucose or UDPgalactose into polymeric material was greatly confused by much of the label being accumulated into glycogen. A

further examination of the problem of simultaneous glucose incorporation into glycogen and wall material has been made and the results will be published elsewhere.

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The butanol-soluble proteins of *Klebsiella aerogenes*

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Abstract

Membranes of *Klebsiella aerogenes* were extracted with acid butanol at pH 4.2 and the solubilized proteins were fractionated by precipitation at 0°C and −20°C. The −20°C precipitate was examined by polyacrylamide gel electrophoresis in sodium dodecyl sulphate.

Several enzyme activities associated with polysaccharide biosynthesis have been identified in the crude −20°C precipitate and an attempt was made to separate the enzymes from other protein. The role of certain of these enzymes in a control mechanism for polysaccharide synthesis in *K. aerogenes* has been postulated.

Introduction

The biosynthesis of peptidoglycan, lipopolysaccharide and exopolysaccharides (capsules and slime) by the Gram-negative bacterium *Klebsiella aerogenes*, involves the formation of oligosaccharide repeating units which are assembled on an isoprenoid alcohol phosphate carrier lipid prior to polymerization (Rothfield and Romeo, 1971; Lennarz and Scher, 1972; Troy *et al.*, 1971). These reactions are catalysed by membrane-bound (particulate) enzymes and solubilization by conventional means has proved difficult (Sutherland and Norval, 1970). Sandermann and Strominger (1972) used acid butanol solubilization and precipitation at low temperature, to purify the highly hydrophobic enzyme isoprenoid alcohol phosphokinase from membranes of *Staphylococcus aureus*. This provided a possible method for extracting other hydrophobic proteins from bacterial membranes.

The solubilization by the acid butanol technique of two glycosyl transferase involved in exopolysaccharide biosynthesis (Lomax *et al.*, 1973) and an iosprenoid alcohol kinase (Poxton *et al.*, 1974) from membranes of *K. aerogenes* have been briefly described. This report describes activities present in the crude butanol-soluble extract, an analysis of the soluble material and the attempted fractionation and purification of the isoprenoid alcohol phosphokinase, which differs in some properties from the corresponding enzyme isolated from the Gram-positive bacterium *S. aureus* (Sandermann and Strominger, 1972).

Materials and methods

A non-mucoid strain of *K. aerogenes* A4 (type 8) was grown and 'membranes' prepared as earlier described (Lomax *et al.*, 1973). Extraction of the butanol-soluble material was by a method similar to that of Sandermann and Strominger (1972). The bacterial 'membranes' (equilavent to approximately 60 g dry weight) were suspended in a final volume of 500 ml 50 mM Tris-HCl buffer, pH 7.3 containing 1 mM $MgCl_2$. This suspension was stirred in acid butanol (120 ml 6 M pyridinium acetate, pH 4.2 + 320 ml butan-l-ol) at room temperature for 40 min. The phases were separated by centrifugation at 6,000 x *g* for 20 min at room temperature and the upper butanol phase was pipetted into a separating funnel containing 250 ml butanol-saturated water. The

residual aqueous material was extracted twice more with 300 ml butanol; the butanol layers, after washing, were combined. The butanol solution was concentrated by rotary evaporation at 25°C until all the azeotrope had distilled over and stored in ice at 0°C for 24–72 h, then centrifuged at 10,000 x *g* for 15 min. The butanol solution held at −20°C in centrifuge tubes for 24 h was centrifuged in a pre-cooled rotor at −20°C by accelerating to 13,000 x *g* then immediately stopped. The crude butanol enzyme was resuspended in butanol and stored at 0°C.

Preparation of polyprenols

Ficaprenol was prepared by the method of Stone *et al.* (1967) from leaves of the decorative rubber plant (*Ficus elasticus*). Bactprenol was extracted from *Lactobacillus plantarum* by the procedure of Thorne and Kodicek (1966). Geraniol and farnesol were purchased from Koch Light Ltd, Colnbrook, Buckinghamshire, England; geranylgeraniol was a gift from Ciba-Geigy, Basel, Switzerland and solanesol from the Tobacco Research Laboratories, Bristol, England.

Assay of polyprenol phosphates

Incubation mixtures were prepared by drying under vacuum: 20 μl butanol-soluble enzyme (350 μg protein), 30 μl Span 20 (0.1% solution in methanol) and the polyprenol. 25 μl of a buffer containing 50 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 100 mM NaH_2PO_4, 600 mM dimethyl sulphoxide, 0.1% 2-mercaptoethanol and 0.3% Triton X 100 were added and mixed with a fine capillary tube. The reaction was started by additon of 0.05 μCi (0.1 μmoles) of ATP-γ-^{32}P in a volume of 1 or 2 μl. The reaction was stopped by either (1) extracting with 4 ml chloroform/methanol (2:1), partitioning with 1 ml saline and running the concentrated organic extract on thin layer plates in di-isobutyl ketone, acetic acid, water (40:25:5) and scanning in a Tracerlab 4π Scanner (Tracerlab Ltd, Weybridge, Surrey, England), or (2) adding 30 μl tetrahydrofuran and running the whole mixture as a 3 cm strip on Whatman 3MM paper (18 x 6 cm) by ascending paper chromatography in isobutyric acid, 1.0 M aqueous ammonia (5:3). Radioactive polyprenyl phosphates which run at the solvent front in this solvent, were either detected in the Tracerlab scanner or cut out and counted in 0.5% PPO (Diphenyloxazole) in toluene in a Packard Tricarb liquid scintillation spectrometer (Packard Instruments Ltd, Caversham, Berkshire, England).

Polyacrylamide gel electrophoresis as modified from Weber and Osborn (1969)

Proteins were solubilized in gel buffer containing 1% sodium dodecyl sulphate (SDS) and 1% 2-mercaptoethanol for 4 h at 37°C or overnight at 20°C with vigorous shaking. For application to gel, 10 μg of protein standard or 100–200 μl of unknown solubilized protein in 10 μl buffer, were mixed with 1 drop of 10% (w/v) sucrose and 1 μl tracking dye (0.05% bromophenol blue).

Ten per cent acrylamide gels with 0.27% *bis*acrylamide were prepared in 65 x 5 mm stoppered glass tubes. Electrophoresis was performed in Quickfit and Quartz (Stone, Staffordshire, England) equipment at a constant current of initially 5 mA per tube until the samples had entered the gel, then at 8 mA until the tracking dye had migrated about 75% through the gel (2.5–3 h).

Staining and destaining

Gels were immersed in (1) to (5) [according to the method of D. F. Wallach (personal communication)]. The solutions were made up as follows: solution (1) 1.25 g Coo-

massie brilliant blue R, 1625 ml water, 625 ml propane-2-ol, 250 ml acetic acid for 6–12 h; solution (2) 125 mg Coomassie blue, 2000 ml water, 250 ml propan-2-ol, 250 ml acetic acid for 6–12 h; solution (3) 63 mg Coomassie blue, 2250 ml water, 250 ml acetic acid for 6–12 h; solution (4) 1000 ml water, 800 ml methanol, 200 ml acetic acid for 4–6 h; and solution (5) 10% acetic acid for 12 h. The mobility of the proteins was calculated as indicated by Weber and Osborn (1969).

Protein was estimated in the presence of SDS by the method of Lowry *et al.* (1951). Phosphorus was determined by the method of Barlett (1959). Thin layer chromatography of the lipids found in association with crude butanol-soluble proteins was performed on silica gel H in chloroform, methanol, ammonia, water (65:25:0.5:3.6).

Amino acids were assayed qualitatively on Whatman number 1 paper developed in the upper phase of butanol, acetic acid, water (4:1:5), by descending chromatography and stained with ninhydrin. Quantitative analysis was performed in a Beckman Model 120 amino acid analyser by Dr R. P. Ambler (Department of Molecular Biology, University of Edinburgh).

Results

Extraction of membranes with acid butanol

Washed bacteria (63.5 g dry weight) were disrupted and membranes prepared and extracted as described in the Methods. Of the 19 g of protein in the crude membrane

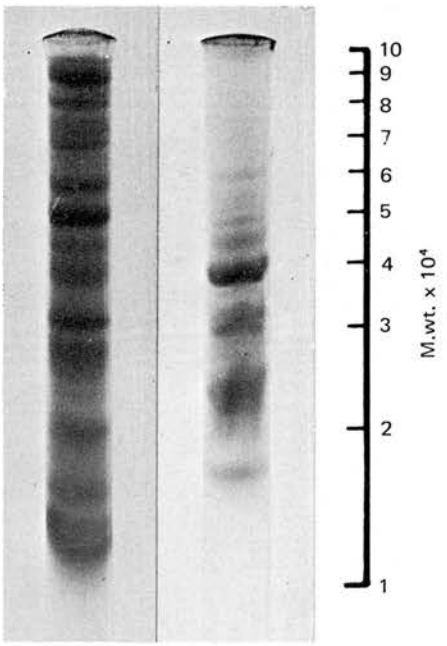

Figure 1 Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulphate of (a) crude membranes of *K. aerogenes*, and (b) the −20°C precipitate after acid-butanol solubilization of the membranes. This was performed as described in Materials and methods. Molecular weights were determined from the following protein standards – bovine serum albumen, deoxyribonuclease, catalase, ovalbumen, lactate dehydrogenase, trypsin, lysozyme, ribonuclease and cytochrome *C*, run under identical conditions.

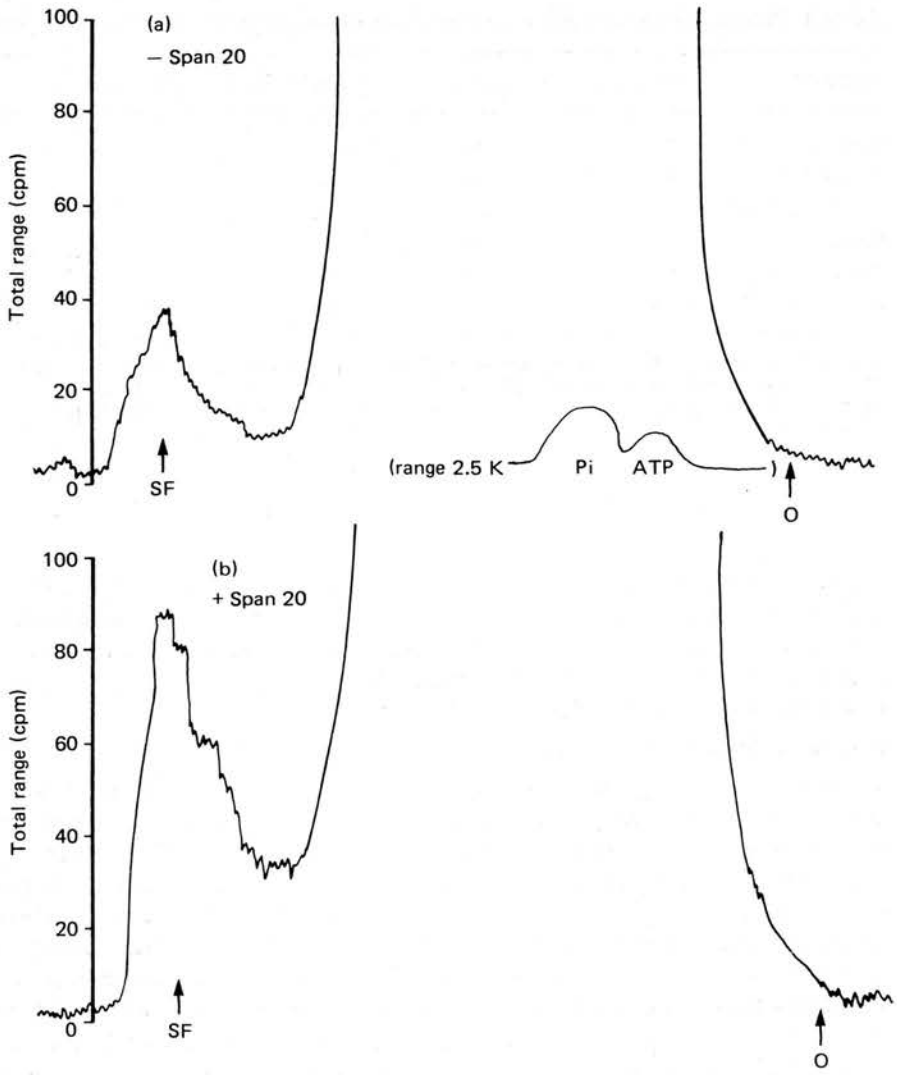

Figure 2 Synthesis of undecaprenyl phosphate. Scans of radioactive product on paper chromatograms. Incubation mixtures were prepared with ficaprenol, crude BSE and 50 mM tris-HCl (pH 8.5) buffer (see text). Span 20 detergent was omitted from (a) but added to (b). After 30 min incubation with ^{32}P-γ-ATP the whole mixture was applied to paper, developed in iso-butyric acid: 1 M aqueous ammonia (5:3) and scanned as described in text, using the Tracerlab 4π scanner with a full scale range of 100 cpm and a time constant of 150 sec. O = origin; SF = solvent front.

Table 1 Phosphokinase activity with isoprenols of varying chain length as substrates

Isoprenol	No. isoprene units	Mol. wt.	P incorp. (pmol)/mg protein/min
Geraniol	2	154	1640
Farnesol	3	222	1790
Geranyl-geraniol	4	290	875
Solanesol	9	630	417
Bactoprenol	11	766	3560
No addition	–	–	30

Incubation mixtures were set up as described in Materials and methods. After 30 min incubation each reaction was terminated by the addition of 30 µl tetrahydrofuran and chromatographed on paper in isobutyric acid:1 M ammonia (5:3). The solvent fronts were cut out and assayed for radioactivity.

pellet, 60 mg (0.32%) was soluble in acid butanol. After the −20°C precipitation, 28 mg crude butanol soluble enzyme (BSE) was obtained (0.15% of the total membrane protein). A comparison between the proteins in the unextracted membranes and those in the butanol soluble fraction was made by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (0.1%) (Figure 1).

Enzyme activities associated with crude BSE

The presence of two glycosyl transferases and a C_{55} isoprenoid alcohol phosphokinase in crude BSE preparations has already been reported and was confirmed. Incubation mixtures prepared as in the Methods were made with and without the detergent Span 20 to test the effect on synthesis of isoprenyl phosphates. After separation of the products by paper chromatography, the results were as shown in Figure 2. It was possible to resolve the large peak shown in Figure 2 into two separate peaks. These corresponded in their mobilities, to inorganic phosphate and unreacted ATP. Several isoprenoid alcohols of chain length less than 11 isoprene units were tested to determine whether they would act as substrates for the phosphokinase present in crude BSE. The results, after 30 min incubation and assay of the material running at the solvent front on paper chromatography (solvent – isobutyric acid: ammonia), are given in Table 1. The identity of the radioactive material was checked by thin layer chromatography of the chloroform/methanol soluble material from duplicate incubation mixtures (Figure 3).

Attempted purification of BSE

Crude BSE (28 mg protein) from *K. aerogenes* membranes was extracted with 3 x 10 ml butanol at room temperature for 20 min with shaking and the pellet removed by centrifugation at 20,000 x *g* for 2 min. Soluble material (fraction 1) was removed and the pellet (fraction 2) retained. Fraction 1 was evaporated with about 5 g of 3 mm diameter glass beads in a round-bottomed flask, then extracted with 25 ml methanol by shaking at room temperature for 1 h. After centrifugation in a bench centrifuge for 5 min the soluble material (fraction 3) was removed. The pellet was sequentially extracted with 25 ml each of 5% butanol in methanol (fraction 4); 10% butanol in methanol (fraction 5); 30% butanol in methanol (fraction 6); 50% butanol in methanol (fraction 7) and

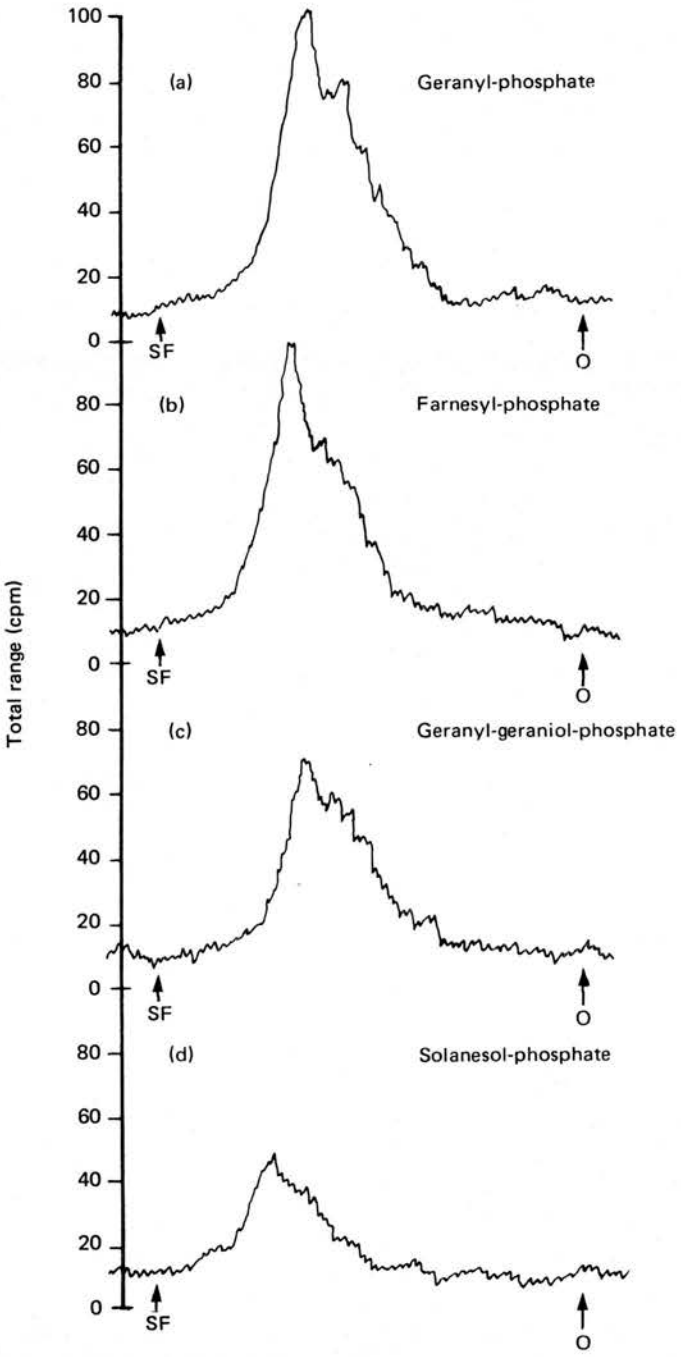

Figure 3 Synthesis of shorter chain length prenyl phosphates. Chloroform/methanol (2:1) extracts of incubation mixtures with (a) geraniol, (b) farnesol, (c) geranyl-geraniol, and (d) solanesol as substrates, were incubated and chromatographed as described in the text (solvent was diisobutylketone:acetic acid:water, 40:25:5). Instrument settings were as for Figure 2. O = origin; SF = solvent front.

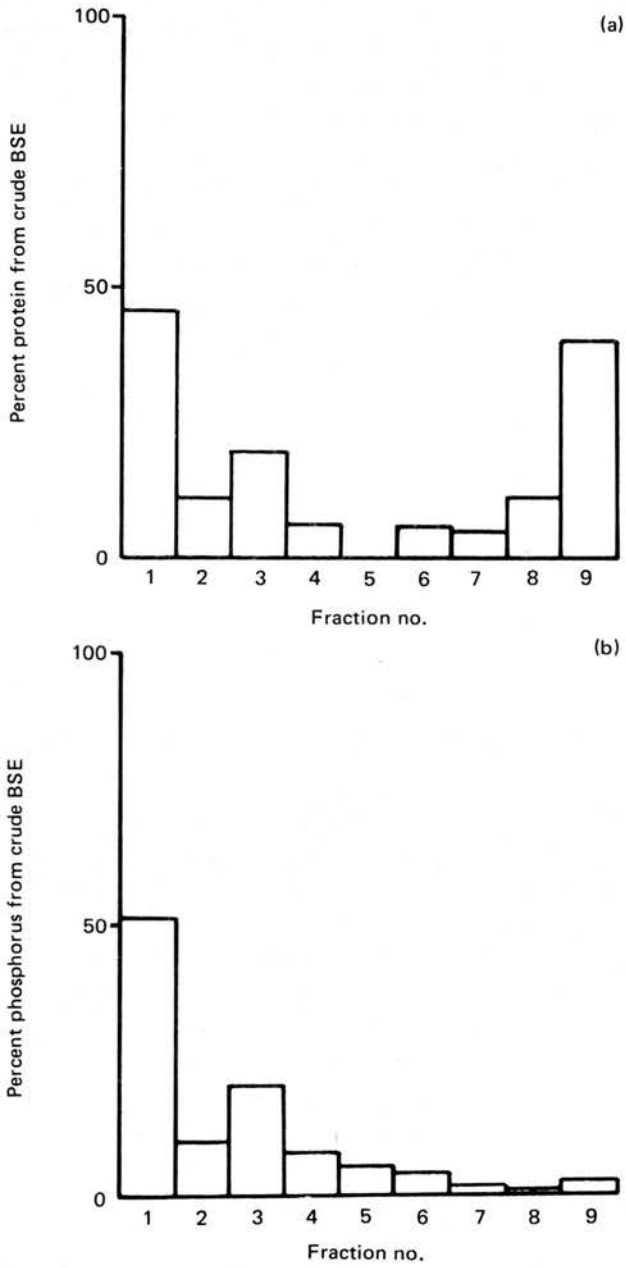

Figure 4 Protein and phosphorus solubilized from crude BSE during attempted purification. Fraction 1, butanol soluble material from crude BSE; fraction 2, butanol insoluble material; fraction 3, methanol soluble material from butanol-solubilized crude BSE; fraction 4, 5% (v/v) butanol in methanol solution from BSE; fraction 5, 10% (v/v) butanol in methanol solution; fraction 6, 30% (v/v) butanol in methanol solution; fraction 7, 50% (v/v) butanol in methanol solution; fraction 8, 100% butanol; fraction 9, residual insoluble material. Procedure as in Results.

finally 25 ml butanol (fraction 8). The pellet was designated fraction 9. Each fraction was analysed for protein, total phosphorus (Figure 4) and isoprenoid alcohol phosphokinase activity (Table 2). An attempt to examine the fractions by polyacrylamide gel electrophoresis was unsuccessful as the large amounts of lipid present interfered.

The activity of the isoprenoid alcohol phosphokinase was apparently lost during the organic solvent fractionation procedure, although Span 20 and cardiolipin were added as possible activators. Attempts to purify this enzyme further were unsuccessful. The failure of this procedure to yield active enzyme indicates that there is a difference in this enzyme from *K. aerogenes* when compared with that from *S. aureus*.

Amino acid analysis of BSE

Although the isoprenoid alcohol phosphokinase could not be fractionated further from the −20°C precipiatate, an examination of the amino acids present would provide an indication of the overall composition of the constituent proteins and also of their hydrophobic nature. Paper chromatography of BSE after hydrolysis in 6 M HCl for 18 h at 100°C showed that the protein contained all the normal amino acids with alanine, glycine, phenylalanine and leucine/isoleucine in the highest concentrations. From quantitative amino acid analysis (Table 3), the content of hydrophobic amino acids as defined by Sandermann and Strominger (1971), is 54.5%.

Discussion

Extraction of membranes of *K. aerogenes* with acid butanol and precipitation at −20°C yielded 0.15% of the membrane protein, containing a number of different proteins (Figure 1). The membrane of Gram-negative bacteria thus contains a number of proteins soluble in butanol and presumably resembling the isoprenoid alcohol phosphokinase

Table 2 Isoprenoid alcohol phosphokinase activity in fractions of crude butanol-soluble enzyme

Fraction	P incorp.(pmol)/mg protein/h
Crude BSE	36,000
1	31,000
2	1,400
3	420
4	1,100
5	0
6	710
7	1,200
8	1,600
9	450

Incubation mixtures were set up as described in Materials and methods. Similar amounts of protein (200–400 μg) from each fraction were added as enzyme, and Span 20, bactoprenol and buffer were added as in text. The whole incubation mixture, after 30 min was treated as in Table 1 and radioactivity at solvent front assayed.

Table 3 Amino-acid composition of the crude butanol-soluble enzyme from *Klebsiella aerogenes*

Amino-acid	nmoles*
Lysine	20
Histidine	9
Arginine	23
Aspartic acid	34
Threonine	26
Serine	28
Glutamic acid	36
Proline	22
Glycine	56
Alanine	66
Half cystine	0
Valine	47
Methionine	14
Isoleucine	31
Leucine	75
Tyrosine	13
Phenylalanine	36

* Results expressed as nmoles of amino-acid per 0.500 A_{280}

from *S. aureus* in their general amino acid composition (Sandermann and Strominger, 1972). Not all these proteins need possess enzymatic activities, but, as we have already identified three distinct enzyme reactions associated with the BSE preparations, more may be present. Attempts to fractionate the crude mixture, using methods applicable to a Gram-positive bacterium, were unsuccessful, and indicate that the prenol kinase may be differently organized within the Gram-negative membrane. Protein, together with phosphatidylethanolamine and cardiolipin was removed during fractionation, but the high content of hydrophobic amino acids (Table 3) amounting to 54.5% compared with the 57.8% reported for the *S. aureus* enzyme apoprotein (Sandermann and Strominger, 1972) may account for the butanol solubility of these proteins.

The incubation mixture for phosphokinase assay contains a large excess of inorganic phosphate. Despite this, incubation mixtures produced ^{32}P-labelled inorganic phosphate. This taken together with the evidence for a reproducible dephosphorylation of isoprenoid alcohol phosphate on prolonged incubation (Poxton *et al.*, 1974), suggests that BSE contains a phosphatase. Slight activity against nitrophenylphosphate was demonstrated, but the specificity of the phosphatase is not known, nor is it certain that the same enzyme dephosphorylates the isoprenyl phosphate. *E. coli* alkaline phosphatase is inactive against polyprenyl phosphates synthesized by *Micrococcus lysodeikticus* (Keanan and Allen, 1974) under conditions where nitrophenyl phosphate is hydrolysed.

Other prenols act as substrates for the phosphokinase with lower efficiency than bactoprenol. Solanesol, the nearest in chain length to bactoprenol of those tested, is

the poorest substrate. This probably due to the isoprene units being in the *trans* configuration; all the other prenols tested have the *cis* configuration at the -OH terminal isoprene unit. Higashi *et al.* (1970) using a wide variety of isoprenoid alcohols as potential acceptors in tests with a phosphokinase from *S. aureus*, reported maximum activity with C_{50} and C_{55} ficaprenols. Solanesol was a very poor acceptor, which did not stimulate the formation of peptidoglycan lipid intermediate when added with ATP to incubation mixtures. The lower phosphorylation of geranylgeraniol than isoprenols of shorter chain length cannot be explained, but thin layer chromatography of replicate incubation mixtures showed peaks of comparable magnitude to the values in Table 1. There may be two different kinases within the crude BSE, one for C_{55} alcohols and one for shorter chain length prenols. In the synthesis of polyprenyl pyrophosphates from Δ^3-isopentenyl pyrophosphate by *M. lysodeikticus* (Allen *et al.*, 1967) and *Salmonella newington* (Christenson *et al.*, 1969), the shorter chain length prenyl pyrophosphates were synthesized by one enzyme and had the longer chain length compounds by a second enzyme. Allen *et al.* (1967) also showed that in *M. lysodeikticus* the shorter chain length pyrophosphates are more susceptible to alkaline phosphatase than are the longer chain length products.

From the results discussed above and the fact that free isoprenoid alcohol has been isolated from *K. aerogenes* (J. A. Lomax, unpublished results) as well as *Streptococcus faecalis* (Umbreit *et al.*, 1972). it seems likely that the control mechanisms postulated by Sandermann and Strominger (1971, 1972) for *S. aureus* are not unique to that bacterium but also exist in *K. aerogenes*. This would provide a useful mechanism for contolling the level of available isoprenoid alcohol phosphate for use in polysaccharide synthesis. If the phosphokinase-phosphatase system controls the gross level of isoprenyl phosphate carrier lipid in the bacteria, a second mechanism may be required to control which polymer – peptidoglycan, lipopolysaccharide or exopolysaccharide – is synthesized.

The control of this system is not yet understood but may depend on spatial separation of the enzymes and substrates involved.

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The Structure of C-Polysaccharide from the Walls of *Streptococcus pneumoniae*

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The well-known immologically active component of pneumococci, C-polysaccharide, is a teichoic acid that can be isolated from the cell walls and purified by Sephadex and ion-exchange chromatography. Further details of the structure of C-teichoic acid were established by chemical degradation, including hydrolysis in acid and alkali, treatment with HF, periodate oxidation and methylation. In addition, the use of ^{13}C n.m.r. has confirmed some of these structural features and resulted in a proposal for the order of substituents, the location of positions of substitution and the configuration of anomeric centres in the repeating unit of the polymer.

The C-teichoic acid (C-polysaccharide) of *Streptococcus pneumoniae* is receiving renewed attention in immunology. It is mitogenic for T lymphocytes; thus, as it is a widespread contaminant in most pneumococcal capsular polysaccharide preparations, its presence might affect the use of the preparations in studies on their effect on the immune response (J. H. Humphrey, personal communication). It possesses choline phosphate haptenic groups and binds both to idiotypic determinants on immunoglobulins produced against other molecules possessing choline phosphate residues, and to certain myeloma proteins with binding sites specific for choline phosphate (Brown & Crandall, 1976; Glaudemans *et al.*, 1977). C-reactive protein, an acute-phase protein that is found in human serum during several pathological conditions including tissue injury, carcinoma and the febrile stages of infections with various micro-organisms, is precipitated by C-teichoic acid. This again probably involves the choline phosphate determinant and complement is consumed *in vivo* (Kaplan & Volanakis, 1974). An homology has been noted between human and rabbit C-reactive proteins, immunoglobulins and histocompatibility antigens by amino acid sequencing (Osmand *et al.*, 1977).

The physiological importance of choline phosphate residues in the cell wall of the pneumococcus is also noteworthy and well documented. The biosynthetic replacement of choline with analogues such as ethanolamine affects the organism in a number of ways; the cells are unable to separate, genetic transformation is inhibited, and the organism becomes resistant to autolysis, phage infection and the lytic action of penicillin and other cell-wall antibiotics. These altered properties are probably the results of defects in the autolytic system of the organism (Tomasz *et al.*, 1975). A knowledge of the structure of C-teichoic acid is therefore important in understanding the nature of its association with immunoglobulin, myeloma, C-reactive protein and also the autolytic enzyme system of the pneumococcus.

Since the first description of a species-specific somatic antigen (fraction 'C') of the pneumococcus by Tillett & Francis (1930) several structural studies have been made, notably by Goebel *et al.* (1943) and Gotschlich & Liu (1967). Brundish & Baddiley (1968) first showed the polymer to be a teichoic acid comprising a repeating unit containing ribitol phosphate, *N*-acetylgalactosamine, *N*-acetyldiaminotrideoxyhexose, choline phosphate and glucose residues. More recently, Watson & Baddiley (1974) studied the action of HNO_2 on the polymer and proposed a partial structure. In the present paper we describe an improved method of preparation and further work on the structure of C-teichoic acid resulting in the clarification of most of the structural features of the molecule.

Experimental

Materials

Streptococcus pneumoniae A.T.C.C. 12213 was grown in 15-litre batches as previously described (Poxton & Leak, 1977). HF (60%, w/w) was purchased from Hopkin and Williams, Chadwell Heath,

Abbreviation used: Dnp, 2,4-dinitrophenyl.

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Essex, U.K. The glucose oxidase reagents were from Sigma Chemical Co., London KT2 7BH, U.K., alkaline phosphomonoesterase was from Boehringer Corp., London BN7 1LG, U.K., and KB^3H_4 from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were purchased from local suppliers.

Analytical methods

Phosphate was determined by the method of Chen *et al.* (1956), choline by the method of Appleton *et al.* (1953), glucose by the method of Dubois *et al.* (1956) and also by the glucose oxidase reagents, hexosamine by the method of Strominger *et al.* (1959) and amino groups by the ninhydrin method (Rosen, 1957). Amino sugar analyses were carried out on a JEOL amino acid analyser.

Methylation analysis, by preparing partially methylated alditol acetates by the method of Jansson *et al.* (1976), was investigated in a Varian 1400 all-glass gas–liquid chromatograph and a Micromass (V. G. Micromass, Altrincham, Cheshire, U.K.) model 12B2 mass spectrometer operating at 70eV and 4kV accelerator voltage. Capillary columns were used, coated with SP1000 resin for derivatives of neutral sugars and SE30 resin for derivatives of amino sugars (both liquid phases were supplied by Phase Separations, Queensferry, Clwyd, U.K.)

Proton-decoupled ^{13}C-n.m.r. data were determined at 22.63 and 50.3MHz in ^2H_2O solution at 32°C. External tetramethylsilane was used as reference.

Immuno-gel diffusion on agarose plates was by the method of Ouchterlony against C-antiserum kindly given by Dr. Michael Heidelberger (New York University Medical Center, New York, NY, U.S.A.).

Paper chromatography and electrophoresis

Both were carried out on Whatman no. 1 paper or, on a preparative scale, on Whatman 3MM paper (washed with 2M-acetic acid and water). Descending paper chromatography was used with the following solvent systems: A, propan-1-ol/aq. NH_3 (sp.gr. 0.88)/water (6:3:1, by vol.); B, ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.).

Electrophoresis was carried out with a Shandon Southern (Camberley, Surrey, U.K.) apparatus, model L24, with the following buffers: C, 8.0% (v/v) formic acid, pH1.8; D, pyridine/acetic acid/water (1:1:38, by vol.), pH4.7; E, pyridine/acetic acid/water (5:2:93, by vol.), pH5.3.

Compounds were detected by the following methods: reducing sugars by the alkaline silver nitrate reagents (Trevelyan *et al.*, 1950), α-glycols by the periodate/Schiff spray reagents (Baddiley *et al.*, 1956), amino compounds by ninhydrin, phosphate esters by the acid molybdate spray (Hanes & Isherwood, 1949) and choline and choline phosphate by the Dragendorf spray reagent (Bregoff *et al.*, 1953).

Preparation of C-teichoic acid

Washed bacteria were suspended in ice-cold water to a concentration of about 30% (w/v) and disrupted for 2.5min in a Braun homogenizer with no. 11 Ballotini beads. Walls and unbroken bacteria were collected by centrifugation at 10000*g* for 30min at 4°C. The walls were carefully removed from above the pellet of unbroken cells and suspended in water. The suspension was heated rapidly to 80°C and kept at that temperature for 3min to destroy autolytic enzymes. The walls were then washed three times in water. Protein and membrane components were removed by pouring a 30% (w/v) suspension of cell walls into an equal volume of boiling 5% (w/v) sodium dodecyl sulphate and stirring for 4h at room temperature (20°C). The cell walls, which were still slightly contaminated, were washed by centrifugation at 10000*g* for 20min in six changes of water at 20°C.

Cell walls were suspended in ice-cold water to a concentration of about 2.5% (w/v) and trichloroacetic acid was added to a final concentration of 10% (w/v); C-teichoic acid was extracted by stirring for 48h at 4°C. The cell walls were removed by centrifugation at 10000*g* for 30min at 4°C and the trichloroacetic acid was removed from the supernatant fluid by six extractions with equal volumes of diethyl ether. Water was removed by rotary evaporation and nucleic acids by fractionation on a stacked column of Sephadex G-25 and G-75 similar to that described by Slabyj & Panos (1973), except that 0.2M-NaCl was used instead of LiCl. The fractions containing phosphorus, but which did not absorb at 260nm, were combined, decreased in volume and desalted on a Sephadex G-25 column (60cm × 1.5cm). A final purification was carried out by ion-exchange chromatography on a column (30cm × 1.5cm) of DEAE-cellulose (Whatman DE-52), eluting with a continuous gradient (0–1M) of pyridinium acetate, pH5.3. C-teichoic acid was eluted at 0.4M; this was freeze-dried.

Results and Discussion

Acid hydrolysis

C-teichoic acid (2mg) was hydrolysed in 2M-HCl for 3h at 100°C in a sealed tube. Acid was removed *in vacuo* over NaOH and the products were examined by paper chromatography in solvents A and B. The following were identified: ribitol, anhydroribitol, galactosamine, glucose, galactosamine phosphate

and isomeric ribitol phosphates. Electrophoresis in buffer C revealed choline, but not choline phosphate.

Molecular proportions of the constituents were determined with both hydrolysed and unhydrolysed material. After hydrolysis (2M-HCl at 100°C for 3h), the proportions of phosphorus/hexosamine/glucose/choline/amino groups were 2:1.8:0.2 (by glucose oxidase):0.66:2.6. Unhydrolysed material had a molar ratio phosphorus/hexose (phenol/H_2SO_4 method) of 2:0.2. A more accurate value of phosphorus/galactosamine was obtained from the amino acid analyser. C-teichoic acid (5mg) was dissolved in 1.2ml of water; 0.5ml samples were adjusted to a final concentration of 4M-HCl and hydrolysed *in vacuo* for 6 and 24h at 100°C. After removing the HCl over NaOH, samples were examined in the analyser. Total galactosamine was calculated by summation of the free galactosamine and the galactosamine phosphate. The remaining 0.2ml of sample was used for phosphorus determination. Molar ratios of phosphorus/galactosamine were 2:0.87 after 6h and 2:0.95 after 24h. This showed that the true ratio of phosphorus/galactosamine was 2:1 and the anomaly in the hexosamine value was probably due to the diaminohexose, which is a known constituent, being decomposed to a pyrrole that reacts with hexosamine reagents. The presence of three amino groups/two atoms of phosphorus agrees with this conclusion.

Alkaline hydrolysis

C-teichoic acid (20mg) was dissolved in 4ml of 1M-NaOH, sealed in a plastic tube and hydrolysed for 3h at 100°C, cooled, desalted through 20ml of Dowex 50 (NH_4^+ form) resin, evaporated to dryness and redissolved in 2ml of water. Less than 5% of the total phosphorus was present as P_i. A portion (1ml) was treated for 16h at 37°C under toluene with alkaline phosphomonoesterase (10μg of Boehringer suspension) at pH9 [adjusted with $(NH_4)_2CO_3$]. In several experiments, 50–60% of the phosphorus was converted into P_i. Thus about half of the phosphorus was phosphomonoester after alkaline hydrolysis.

The product of alkaline hydrolysis was hydrolysed in acid (2M-HCl, 3h at 100°C) before and after the phosphatase treatment. With solvents A and B, it was found that before phosphatase treatment ribitol, anhydroribitol, galactosamine, ribitol phosphates and galactosamine phosphate were present. After phosphatase treatment, all but ribitol phosphates were present and the amount of ribitol appeared to have increased. This suggests that the ribitol is attached to a phosphomonoester and galactosamine to a diester after the alkaline hydrolysis.

If choline had been produced in the alkaline hydrolysis, it would have been bound to the Dowex 50 resin during desalting. In a control experiment, choline phosphate was treated with 1M-NaOH for 3h at 100°C. After cooling and neutralization with dilute HCl, both organic phosphate and P_i were determined. No P_i was produced, but electrophoresis of the products showed that choline phosphate had been destroyed and a smell resembling trimethylamine was noted. Presumably choline phosphate undergoes a Hofmann elimination to trimethylamine and possibly vinyl phosphate under the experimental conditions. Similarly, when C-teichoic acid was hydrolysed with alkali a characteristic smell of a tertiary amine was observed. The phosphate associated with the choline residue remained as a diester bound to galactosamine. When the hydrolysate was subsequently hydrolysed with acid, chromatography in solvent B revealed a fast-moving product that was not characterized, but was probably derived from the vinyl group attached through the phosphodiester to galactosamine.

Reaction with 1-fluoro-2,4-dinitrobenzene

To detect the diaminohexose, C-teichoic acid was treated with fluorodinitrobenzene to dinitrophenylate the free amino groups. The reaction was carried out by the method of Distler *et al.* (1966); 12mg of material was used and the final purification was by dialysis in several changes of water for 24h. The resultant dinitrophenylated material (Dnp-C), and C-teichoic acid itself, were examined with C-antiserum on a gel-diffusion plate; a sharp yellow precipitin band was obtained continuous with the band given by the C-teichoic acid.

The number of dinitrophenyl groups was determined at A_{360} and compared with a standard curve derived from ε-dinitrophenyl-lysine. A molar ratio of phosphorus/dinitrophenyl groups of 2:0.76 was found, i.e. about one free amino group to two phosphates. Acid hydrolysis (4M-HCl, 4h at 100°C) of Dnp-C (2mg) and electrophoresis in buffer D for 30min at 55 V·cm^{-1} gave a single yellow product that strained with the ninhydrin reagent and migrated about twice as far as dinitrophenyl-lysine. This was similar to the properties observed by Distler *et al.* (1966) for the Dnp-diaminohexose.

Degradation with HF

HF (60%, w/w) converts phosphomono- and phosphodi-esters into P_i and phosphorofluoridates without significant hydrolysis of glycosidic linkages (Lipkin *et al.*, 1969). To protect the sensitive diaminohexose, the C-teichoic acid (20mg) was dinitrophenylated as described above and a dry sample in a plastic tube was treated with 1ml of 60% (w/w) HF at 0°C for 16h. After neutralization with 60ml of Dowex 2

($CO^2{}_3^-$ form) resin by the method of Anderson *et al.* (1977), the resulting material was examined by electrophoresis in buffer E; a single yellow spot remained at the origin, and a spot corresponding to choline was detected by the Dragendorf spray. On paper chromatography in solvent A a single yellow spot, $R_{ribitol}$ 1.3, was seen; in solvent B it had $R_{glucose}$ 1.6. The yellow material was purified by preparative electrophoresis in buffer E on Whatman 3MM paper, where it remained at the origin. It was eluted in water and hydrolysed with acid. Under vigorous conditions (4M-HCl for 6h at 100°C) a yellow spot was observed on electrophoresis in buffer D corresponding to Dnp-diaminohexose. Galactosamine, ribitol, anhydroribitol and glucose were also observed on paper chromatograms. Under less vigorous conditions (2M-HCl for 3h at 100°C) an additional yellow spot was detected, migrating 1.5 times as far as the Dnp-diaminohexose; this was a disaccharide of galactosamine and the diaminosugar, both of which had been de-*N*-acetylated. This disaccharide is discussed below.

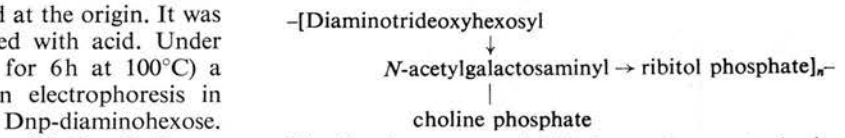
When the yellow product obtained by treatment of Dnp-C with HF was treated with 0.1M-HCl at 100°C for 30min and the mixture examined by paper chromatography, ribitol and glucose were detected together with two yellow products in roughly equal amounts; one had $R_{glucose}$ 1.1 (solvent B), which was the same as the starting material, and the other had $R_{glucose}$ 1.5. This second product was eluted from the paper and a portion hydrolysed in 2M-HCl at 100°C for 3h; the major products were Dnp-diaminohexose ($R_{glucose}$ 1.7), galactosamine (in solvent B) and a yellow component with $R_{glucose}$ 1.3, which was de-*N*-acetylated starting material (this was identical with the disaccharide described above). After more vigorous hydrolysis (4M-HCl for 6h at 100°C) only galactosamine and Dnp-diaminohexose were observed.

Reduction of the disaccharide with KB^3H_4

The disaccharide (1mg) prepared as above was treated with 0.2ml of 0.2M-KBH_4 [containing 14.8MBq (0.4mCi) of ^3H] for 2h in a fume cupboard. Excess of BH_4^- was destroyed by adding 50% (v/v) acetic acid dropwise until the pH was below 5. The solution was freeze-dried, the products were treated with 2M-HCl for 3h at 100°C and, after desiccation over NaOH, dissolved in 0.5ml of water, desalted through Dowex 50 (H^+ form) resin and eluted with 20ml of 1M-NH_4OH. Borate was removed by distillation with methanol (5 times) and Cl^- and reducing sugars were removed by passage through 2ml of Dowex 2 (OH^- form) resin. The colourless solution contained a radioactive alditol that was indistinguishable from galactosaminitol in solvent A and on electrophoresis in buffer E. This confirmed that galactosamine was the reducing component of the disaccharide.

Order of substituents in the repeating structure

The present results, together with those obtained before (Brundish & Baddiley, 1968; Watson & Baddiley, 1974) enable a partial structure to be derived for the main repeating unit, i.e.:



The diaminosugar and ribitol were known to be in the polymer chain because of their respective destruction by HNO_2 (Watson & Baddiley, 1974) and by periodate (Brundish & Baddiley, 1968), causing simultaneous fission of the chain. Moreover, *N*-acetylgalactosaminylribitol was characterized as a degradation product (Watson & Baddiley, 1974). The isolation in the present work of a disaccharide containing the diaminosugar attached to a reducing galactosamine confirms the above sequence.

Although the study by Watson & Baddiley (1974) and the present work demonstrate the presence of glucosyl substituents in the polymer, the amount is insufficient for one glucose to each repeating unit. It is likely then that either only a few of the repeating units are glucosylated or that the glucose is localized at the end of the chain. It is not attached through a phosphodiester at its 1-position because it remains attached to the unit obtained by HF treatment, and reduction of this with $B^3H_4^-$ gives no labelled glucitol residue. Watson & Baddiley (1974) suggested that the glucosyl substituent might be associated with phosphate, since a product tentatively identified as glucitol phosphate had been detected, but it now seems likely that this identification was incorrect. Although the location of glucose is still inadequately established, the methylation and n.m.r. studies described below indicate the presence of a glucosyl-glucosyl (β-isomaltosyl) substituent.

Periodate oxidation

Substitution of the galactosamine residue was examined by the action of periodate. Aqueous solutions (1ml) of polymer and the Dnp/HF product (1mg of each) were mixed with 5mM-$NaIO_4$ (1ml) in 0.1M-acetate buffer at pH4.5. After 16h at room temperature under toluene, excess of periodate was destroyed by adding ethylene glycol (5μl), solvent removed *in vacuo* and the residue hydrolysed in 4M-HCl for 6h at 100°C. After removal of acid over NaOH *in vacuo* galactosamine was determined with the autoanalyser. In neither experiment was a decrease in aminosugar detected after the periodate

treatment, whereas galactosamine itself was completely destroyed under similar conditions.

It follows that the diaminohexose must be attached to the 3- or 4-position on the *N*-acetylgalactosamine residue. If it had occupied the 6-position then the removal of the choline phosphate residue by HF from the 3- or 4-position would have created an oxidizable glycol structure.

There is strong justification for the assumption that the phosphodiester substituent on ribitol must occupy the D-5-position. All of the many ribitol teichoic acids examined are derivatives of D-ribitol 5-phosphate that, in the form of CDP-ribitol, is the biosynthetic precursor of these compounds. It follows that, as the ribitol residues in the polymer are destroyed by periodate, causing chain fission, the *N*-acetylgalactosaminyl substituents should occupy the D-1- or -2-positions on ribitol.

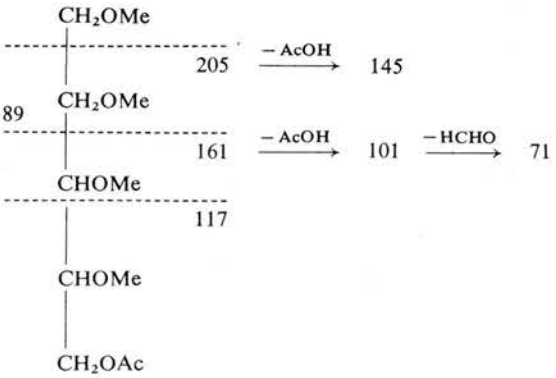
Preparation of N-acetyl/HF product

For the following methylation and ^{13}C-n.m.r. studies the HF product was prepared from fully *N*-acetylated C-teichoic acid. This was preferable to using dinitrophenylated polymer.

The C-teichoic acid (120mg) was dissolved in 10ml of water and 2ml of saturated $NaHCO_3$ solution was added. This was cooled in ice and 2ml of fresh ice-cold aq. 5% (v/v) acetic anhydride was added. Incubation was for 25min at room temperature and then excess of acetic anhydride was destroyed by heating to 100°C for 3min. The *N*-acetylated polymer was first desalted through a 20ml column of Dowex 50 (NH_4^+ form) resin, decreased in volume, then further purified by passage down a column (60cm × 1.5cm) of Sephadex G-25 and finally freeze-dried in a plastic tube. A yield of 114mg was obtained. This was treated with 60% (w/w) HF as for the Dnp-polymer. After HF treatment, the products were again *N*-acetylated as above. The oligosaccharide products were fractionated on a column (60cm × 1.5cm) of Sephadex G-10 and the highest-molecular-weight fraction was freeze-dried. A yield of 25mg of pure *N*-acetylated HF product was obtained, which contained ribitol, *N*-acetylgalactosamine, *N*-acetyldiaminohexose and glucose.

Methylation studies

The position of substitution on ribitol and the nature of the glucosyl substituents were studied by methylation followed by hydrolysis, reduction, acetylation and examination by g.l.c.–mass spectrometry. The method of Hakomori (1964) as developed by Jansson *et al.* (1976) was used with minor modifications. The *N*-acetyl/HF product (4mg) from C-teichoic acid was dissolved by ultrasonic treatment in dry dimethyl sulphoxide (2ml) under N_2. Preparation of the partly methylated alditol acetates was as described (Björndal *et al.*, 1967; Jansson *et al.*, 1976), except that hydrolysis was with 2M-HCl for 3h at 100°C, followed by removal of acid over NaOH *in vacuo*. The partly methylated alditol acetates of neutral sugars were examined on a column of SP1000 resin, the results being given in Table 1. Products (C)–(F) were identified by comparing their mass spectra with the published data on authentic standards (Jansson *et al.* 1976). Product (A) had a similar retention time (approx. 7min) to the isomeric 4-*O*-acetyl-1,2,3,5-tetra-*O*-methylribitol and ions of *m/e* 71, 89, 101, 117, 145 and 161 were identified. These were formed as follows:



The mass spectrum was significantly different from that of the above-mentioned isomer. Product (B) had a slightly longer retention time than did authentic 2,3,5-tri-*O*-methyl-1,4-anhydroribitol, and both the molecular ion (*m/e* 204) and the ion from the ring (positions C-1 to C-4) with *m/e* 131 were identified. It follows that in the *N*-acetyl/HF product ribitol is monosubstituted at a terminal position, and consequently the *N*-acetylgalactosaminyl substituent must occupy that position.

The structure of the glucitol derivatives arising from the glucosyl substituents was also established. 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol must represent a glucose residue at a non-reducing terminus, and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol represents a glucose substituted at its 1- and 6-positions. These results agree with the presence of a glucosyl(1 → 6)glucosyl substituent in the polymer and this is supported by the n.m.r. data.

Although partially methylated derivatives from the amino sugars might have been expected, in fact g.l.c.–mass spectrometry studies of these proved unsatisfactory. They are known to be difficult to investigate by this method and a very complex profile was obtained, probably because of their poor volatility and complex fragmentation in the mass spectrometer arising from their thermal instability.

Methylation of C-teichoic acid itself seemed less

Table 1. *Partially methylated products from neutral sugars obtained from C-teichoic acid and its N-acetyl/HF product*
Samples were examined on a capillary column of SP1000 resin and the temperature was controlled between 100 and 200°C at 2°C/min. Abbreviations: +++, >30%; ++, 10–30%; +, 5–10%; tr, <5% of the sum of peak heights.

Derivative	Present in: C	Present in: N-Ac-HF	Relative retention time
(A) 5-*O*-Acetyl-1,2,3,4-tetra-*O*-methylribitol	—	+++	0.39
(B) 5-*O*-Acetyl-2,3-di-*O*-methyl-1,4-anhydroribitol	+++	tr	0.56
(C) 1,4-Di-*O*-acetyl-2,3,5-tri-*O*-methylribitol	++	tr	0.73
(D) 1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methylribitol	tr	tr	0.76
(E) 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol	+	++	1.00
(F) 1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol	+	+	1.25

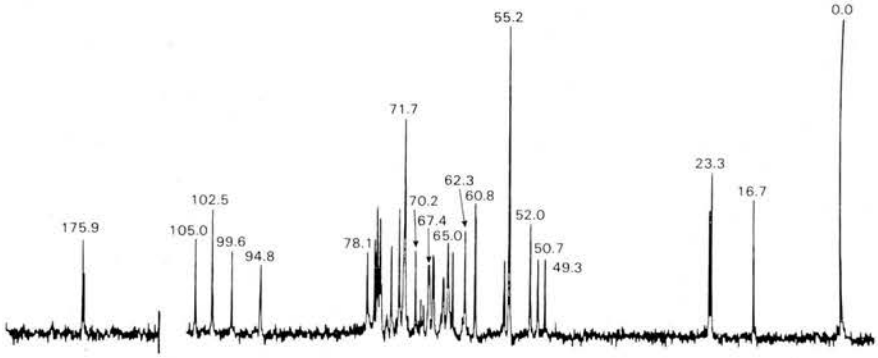


Fig. 1. *Proton decoupled ^{13}C n.m.r. spectrum of C-teichoic acid at 50.3 MHz in ^2H_2O*

attractive, since the presence of hydroxy groups in the vicinity of the phosphodiester linkages might be expected to facilitate cyclization during methylation and consequent fission of diesters. This problem has been long recognized in nucleic acid chemistry. Nevertheless, an unpublished report cited by Conrad (1972) suggested that methylation by the Hakomori (1964) method might leave phosphodiester groups intact. Consequently, C-teichoic acid (7mg) was methylated and products were hydrolysed as described for the *N*-acetyl/HF product. The hydrolysate was treated with alkaline phosphatase (10μg in 1ml) under toluene after adjustment to pH9 with aq. NH_3. After 16h at 37°C, all of the phosphorus had been converted into P_i. Reduction and acetylation was followed by examination with g.l.c.–mass spectrometry. Results are given in Table 1; these suggest that fission of phosphodiester linkages might well have occurred, giving anhydroribitol derivatives. However, even in this case the glucitol derivatives were easily detected, suggesting the presence of the glucosyl-(1→6)glucosyl substituent in the polymer.

^{13}C *n.m.r. spectroscopy*

Proton-decoupled ^{13}C n.m.r. spectra of C-teichoic acid and of its *N*-acetyl/HF product were determined in ^2H_2O solution. Fig. 1 shows the spectrum (50.3 MHz) of C-teichoic acid and Table 2 gives the calculated and observed chemical shifts relative to internal tetramethylsilane together with the assignments for both the polymer and the *N*-acetyl/HF product. Chemical-shift values were calculated from the reported values of the individual units by allowing increments for the formation of glycosidic and phosphodiester bonds (see Table 3). The spectrum of the polymer, although not fully interpreted (owing in part to the lack of data for 2-acetamido-4-amino-2,4,6-trideoxyhexose), nevertheless provided important structural information, including positions of glycosylation, anomeric configurations and confirmation of the nature of the amino functions.

Two separate signals were observed at low field (approx. 175 p.p.m.) and high field (approx. 23 p.p.m.) portions of the spectrum of the polymer, readily attributable to the carbonyl and methyl carbons of two acetamido functions. In addition, three signals characteristic of the $-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{N}}$ group were observed at 49.3, 50.7 and 52.0p.p.m., that at 49.3p.p.m. being assigned to C-4 of the diaminotrideoxyhexose on the basis of the difference observed for this region in the spectrum of the repeating unit in which the amino

Table 2. *Calculated and observed ^{13}C chemical shifts (in p.p.m. from external tetramethylsilane) with assignments for: (i) C-teichoic acid polymer, concentration 100 mg/ml, and (ii) C-teichoic acid repeating unit, concentration 23 mg/ml*

The observed chemical shifts are from spectra measured at 22.63 MHz in ^2H_2O solutions at 32°C. C, D, G, I and R refer respectively to choline, 2-acetamido-4-amino-2,4,6-trideoxyglucose, *N*-acetylgalactosamine, isomaltose and ribitol moieties; a, unsubstituted with choline phosphate at C-6 of GalN or with isomaltose at C-3 of diaminotrideoxyhexose; b, substituted with choline phosphate at C-6 of GalN or with isomaltose at C-3 of diaminotrideoxyhexose; ^{31}P–^{13}C coupling constants were not determined.

(i) C-teichoic acid polymer

Assignment		>C=O	I1	G1	I1′	D1	G4	I3	D3b	I5	G5a	I3′	I2	G5b	I5′	I2′	R3	R1	R2	I4
Calculated		~175	104.7	103.5	99.4	—	78.0	77.7	—	75.9	75.3	75.0	74.9	74.8	73.8	73.3	72.9	72.2	71.9	71.3
Observed	175.9	175.5	105.0	102.5	99.6	94.8	—	78.1	76.8	76.5	—	76.1	75.5	74.0	—	—	—	—	72.6	71.7

Assignment	I4′	R4	G3	R5	I6	C2	G6b	D5a	D5b	I6′	G6a	C1	$(CH_3)_3\overset{+}{N}$–	G2	D2	D4	$CH_3-\overset{\overset{O}{\Vert}}{C}$	D6
Calculated	71.3	71.3	71.3	67.8	67.4	66.7	65.6	—	—	62.5	62.2	61.2	54.8	53.9	—	—	~23	—
Observed	70.2	—	—	68.0	67.4		56.7	65.0	64.4	62.3	—	60.8	55.9	55.2	52.0	50.7	49.3	23.5	23.3	16.7

(ii) C-teichoic acid *N*-acetyl/HF product

Assignment		>C=O		I1	G1	I1′	D1	G4	I3	D3b	I5	G5	I3′	I2	I5′	I2′	R3
Calculated		~175		104.7	103.5	99.4	—	78.0	77.7	—	75.9	75.3	75.0	74.9	73.8	73.3	72.9
Observed	176.4	175.8	175.5	105.0	102.4	99.6	94.9	—	78.1	—	—	76.8	76.4	74.1	—	73.1	—

Assignment	R4	R1	R2	I4	I4′	G3	I6	D5a	D5b	R5	I6′	G6	D4	G2	D2	$CH_3-\overset{\overset{O}{\Vert}}{C}$	D6
Calculated	72.9	72.2	71.9	71.3	71.3	71.3	67.4	—	—	63.2	62.5	62.2	—	53.9	—	~23	
Observed	—	71.7	70.7	—	69.6	—	67.6	67.3	64.9	63.6		62.2	54.3	51.9	50.5	23.1	16.6

Table 3. *^{13}C n.m.r. chemical shifts (in p.p.m. from tetramethylsilane) of components of C-teichoic acid with increments expected for substitution, thereby giving the calculated chemical shift values for (i) the polymer and (ii) the N-acetyl/HF product*

Increments are given the following values: +7 p.p.m. for the formation of a glycosidic bond at the anomeric carbon, +9 p.p.m. for the formation of a glycosidic bond at other carbons, −1 p.p.m. for the introduction of an adjacent bond (Hamer & Perlin, 1976); +4.6 p.p.m. for the formation of a phosphodiester; −1.6 p.p.m. for adjacent phosphodiester in ribitol (Branefors-Helander *et al.*, 1977); +3.4 p.p.m. for the formation of a phosphodiester at position C-6, −0.5 p.p.m. for an adjacent phosphodiester in hexopyranosides (Bundle *et al.*, 1974). (a) Unsubstituted with choline phosphate at C 6 of GalN; (b) substituted with choline phosphate at C-6 of GalN. Values for the reported shifts were from Usui *et al.* (1973) for isomaltose, Branefors-Helander *et al.* (1977) for ribitol, Bundle *et al.* (1973) for *N*-acetylglucosamine and Johnson & Jankowski (1972) for choline chloride.

Isomaltose (I)												
Carbon	1	2	3	4	5	6	1′	2′	3′	4′	5′	6′
Reported shift	97.7	75.9	77.7	71.3	75.9	67.4	99.4	73.3	75.0	71.3	73.8	62.5
Increment	+7	−1	0	0	0	0	0	0	0	0	0	0
Calculated shifts	104.7	74.9	77.7	71.3	75.9	67.4	99.4	73.3	75.0	71.3	73.8	62.5
Ribitol (R)												
Carbon	1	2	3	4	5							
Reported shift	63.2	72.9	72.9	72.9	63.2							
Increment	+9	−1	0	−1.6	+4.6							
Calculated shift	72.2	71.9	72.9	71.3	67.8							
Increment	+9	−1	0	0	0							
Calculated shift	72.2	71.9	72.9	72.9	63.2							
N-Acetylgalactosamine (G)												
Carbon	1	2	3	4	5	6	>C=O	CH_3–				
Reported shift	96.5	54.9	72.3	69.0	76.3	62.2	175.8	23.4				
Increment	+7	−1	−1	+9	(a)−1 (b)−1.5	(a) 0 (b)+3.4	0	0				
Calculated shift	103.5	53.9	71.3	78.0	(a) 75.3 (b) 74.8	(a) 62.2 (b) 65.6	175.8	23.4				
Increment	+7	−1	−1	+9	−1	0	0	0				
Calculated shift	103.5	53.9	71.3	78.0	75.3	62.2	175.8	23.4				
Choline chloride (C)												
Carbon	1	2	CH_3									
Reported shift	56.6	68.3	54.8									
Increment	+4.6	−1.6	0									
Calculated shift	61.2	66.7	54.8									

function was acetylated. The highest field signal (16.7 p.p.m.) was clearly due to the 6-deoxy group of the same moiety. In the anomeric region of the spectrum, four separate signals were observed; two of these (105.0 and 99.6 p.p.m.), of equal area, were about 0.6 times that of either of the others (102.5 and 94.8 p.p.m.). (Quantification of components on the basis of peak area may be inaccurate because of variations in saturation effects, nuclear Overhauser enhancement etc.) The former pair of resonances were in close agreement with the expected values (104.7 and 99.4 p.p.m.; Usui *et al.*, 1973) for C-1 and C-1′ of a β-isomaltoside but significantly different from those (103.8 and 100.3 p.p.m.) expected for an α-gentiobioside and were assigned accordingly. Of the remaining anomeric signals, that at 102.5 p.p.m. was close to the calculated chemical shift of a β-*N*-acetylgalactosaminyl group, and indeed C-1 of such a group has been observed at this value in several polysaccharides (Hamer & Perlin, 1976; R. Lifely, E. Tarelli & J. Baddiley, unpublished work) The remaining signal in this region was assigned to the anomeric carbon of the diaminotrideoxyhexose, the chemical shift (94.8 p.p.m.) supporting the α-configuration.

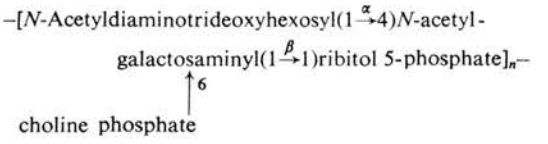
Resonances caused by the choline phosphate substituent could be assigned to those observed at 55.2, 55.9, 60.8 and 68.0 p.p.m., in good agreement with the calculated values, the signals in the region of 55 p.p.m. being assigned to the trimethylammonium group, as has been proposed (Bennett & Bishop, 1977). This set of resonances was essentially absent from the spectrum of the *N*-acetyl/HF product.

The intermediate section of the spectrum (70.2–78.1 p.p.m.) was difficult to assign unequivocally to individual carbon atoms; however, it was significant that after the anomeric signals the next highest chemical shift observed for either the polymer or the *N*-acetyl/HF product was that at 78.1 p.p.m. Two important points arise from this. (i) Position C-3 of

the *N*-acetylgalactosamine residue cannot be involved in a glycosidic linkage since, had this been so, a signal for this carbon at chemical shift 81–82 p.p.m. would have been present; this value would be expected both from calculation and from studies with chondroitins A, B and C and similar polysaccharides containing (1→3)-linked β-*N*-acetylgalactosamine residues (Hamer & Perlin, 1976; R. Lifely, E. Tarelli & J. Baddiley, unpublished work). Therefore in C-teichoic acid the linkage of the diaminotrideoxyhexose must be to the C-4 position of *N*-acetylgalactosamine (bearing in mind the results obtained from the periodate oxidation study). (ii) Ribitol is most probably substituted at its C-1 position by *N*-acetylgalactosamine, since if a secondary carbon of the pentitol were glycosylated then a signal for this carbon atom would have been expected at a chemical-shift value above 80 p.p.m., both from calculation and from studies on similar systems (Branefors-Helander *et al.*, 1977). Thus 4-*O*-β-D-glucopyranosylribitol and related teichoic acids give respectively resonances at 81.7 and 80.1 p.p.m. attributable to position C-4 of the ribitol moiety. The signals observed in the region 70.2–78.1 p.p.m. can then be assigned generally as shown in Table 2.

Structural conclusions from chemical and n.m.r. data

It is possible to propose the following detailed structure for the main chain in C-teichoic acid:



From the chemical evidence already presented, the choline phosphate could occupy either position C-3 or C-6 of *N*-acetylgalactosamine. It is probable, however, that this group is substituted at position C-6 of the amino sugar since a resonance was observed at 65.7 p.p.m. in the spectrum of the polymer, which is in accord with the chemical shift expected for a phosphorylated primary carbon of a hexose (Bundle *et al.*, 1974). Further, a signal at this value was absent from the spectrum of the *N*-acetyl/HF product that, in addition, gave a resonance of increased relative intensity at 62.2 p.p.m. (unsubstituted hydroxymethyl carbon), as would be expected after removal of the phosphodiester.

The remaining feature is the location of the β-isomaltose residue. The low glucose content of the polymer suggests that the residue does not contribute to the main repeating structure, but might be located at one end of the chain. The substitution of isomaltose at a secondary position on ribitol is unlikely for reasons similar to those proposed above in connection with the *N*-acetylgalactosaminylribitol linkage. Moreover, the methylation study indicates that the ribitol in the *N*-acetyl/HF product is only monosubstituted. A more likely possibility is that position C-3 of the chain terminal diaminotrideoxyhexose is glycosylated with β-isomaltose, although in the absence of ^{13}C chemical-shift data on this residue no more than a tentative suggestion can be made. Nevertheless, if a glycosidic linkage of this nature were present, then the chemical shift of the carbon involved in the linkage would be expected to be sufficiently low (owing to the shielding effect of the adjacent amino function) as to be within the range of chemical shifts observed. A further possibility is that position C-6 of those galactosamine residues that do not have a choline phosphate substituent is substituted by an isomaltose residue. The present data do not enable us to distinguish between these possibilities.

The spectrum of the *N*-acetyl/HF product (Table 2) agrees with the structure proposed for the polymer; the differences are accounted for by the absence of phosphate substituents in the former. It is noteworthy that both give signals corresponding to four anomeric centres in glycosyl substituents; these are the two amino sugars and two glucose residues.

Disregarding the isomaltose residue, and assuming that the stereochemistry of the ribitol phosphate is the same as that in CDP-ribitol, the structure of C-teichoic acid is given in Fig. 2. The chain length of the polymer prepared by us has been examined by measuring the choline phosphate-binding sites with a specific antiserum (Glaudemans *et al.*, 1977). It was concluded that there were 4–5 binding sites/molecule, and as about 66% of the *N*-acetylgalactosamine residues in the polymer are substituted with choline phosphate the chain would comprise about 8 repeating units. From the amount of glucose in the polymer a chain of this length would possess about 1 isomaltose residue. It is possible, however, that in the wall itself the teichoic acid might be larger, as the preparations examined were extracted from walls with trichloroacetic acid and this might well have caused a decrease in chain length through hydrolysis.

There is an apparent anomaly in the molar ratio of glucose/phosphorus, the published values being 0.36:2 (Brundish & Baddiley, 1967), 0.74:2 (Brundish & Baddiley, 1968) and 0.2:2 (the present paper), whereas the ^{13}C-n.m.r. data might seem to suggest a yet higher value for glucose. This last evidence should be discounted, however, as peak area is not necessarily proportional to the amount of that carbon atom present owing to differences in shielding of different carbon atoms. In many determinations of the glucose/phosphorus ratio in several different preparations of C-teichoic acid, and by two different methods, we consistently obtained values of about 0.2:2. The present method for the preparation of C-teichoic acid gives a much purer product than hitherto and this might be reflected in the values obtained for

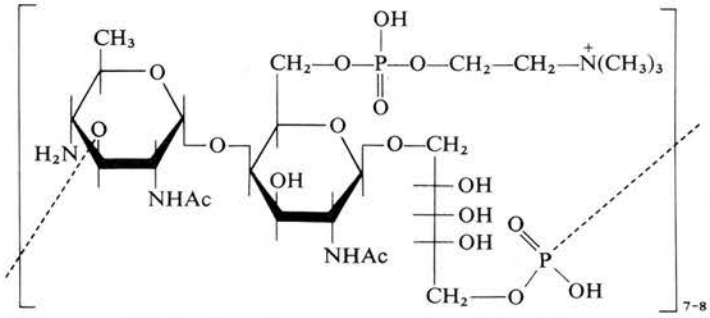


Fig. 2. *Repeating structure in C-teichoic acid*
The isomaltose substituent is not shown.

glucose. It is also possible that culture conditions might influence the amount of substitution by glucose.

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Journal of General Microbiology (1977), **100**, 23–29
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The Biosynthesis of a Choline Nucleotide by a Cell-free Extract from *Streptococcus pneumoniae*

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SUMMARY

Choline, a component of the wall teichoic acid of *Streptococcus pneumoniae*, was converted to cytidine diphosphocholine via choline phosphate by enzymes which were identified in cell-free extracts of the pneumococcus. The first enzyme, choline kinase, was investigated in some detail. It appeared to have a pH optimum of 7·3 to 7·4 and was stimulated by Mg^{2+}. Kinetic studies gave an apparent Michaelis constant (K_m) for ATP of 1 mM, and for choline of 0·19 mM, with V_{max} values of 3 nmol min^{-1} (mg protein)$^{-1}$ and 0·5 nmol min^{-1} (mg protein)$^{-1}$ respectively. The second enzyme, CDPcholine pyrophosphorylase was specific for CTP and had a requirement for Mg^{2+} with an optimum at 7 mM.

INTRODUCTION

Choline, a nutritional requirement of *Streptococcus pneumoniae*, is essential for the normal functioning of the bacterium (Rane & Subbarow, 1940; Tomasz, 1968). It is utilized as a structural component of the wall (C-) teichoic acid (Tomasz, 1967; Brundish & Baddiley, 1968) and of the pneumococcal Forssman antigen (Briles & Tomasz, 1973) but not in phospholipid biosynthesis. Structural analogues of choline, such as ethanolamine, can replace it as a growth requirement but several properties of the organism then become altered, e.g. (i) the daughter cells become unable to dissociate completely, forming chains of several hundred cells, (ii) the bacterium becomes resistant to both autolysis and detergent-induced lysis, and (iii) it is unable to undergo genetic transformation (Tomasz, 1968; Tomasz *et al.*, 1975).

This study reports the biosynthesis of cytidine diphosphocholine (CDPcholine) from choline in a cell-free system of *S. pneumoniae*. A similar pathway has been proposed in brewers' yeast which utilizes choline as a component of phosphatidylcholine (lecithin), one of the major phospholipids of this organism. This first enzyme of the pathway in yeasts, the choline kinase, has been investigated in some detail (Brostrom & Browning, 1973) and is compared here with the pneumococcal enzyme.

METHODS

Materials. [*methyl*-^{3}H]Choline chloride was purchased from The Radiochemical Centre, Amersham. [*methyl*-^{3}H]Choline phosphate was synthesized chemically from [*methyl*-^{3}H]-choline chloride by the method of Riley (1944), except that it was purified by preparative electrophoresis on Whatman 3MM paper in buffer B instead of by precipitation and

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recrystallization of the calcium salt. Unlabelled choline chloride and CDPcholine were from Sigma, and choline phosphate from Boehringer.

Growth of organism. Streptococcus pneumoniae ATCC12213 was maintained on blood agar slopes at 4 °C and subcultured at monthly intervals.

For preparation of the crude enzyme, batches of the organism (15 l) were grown in a laboratory fermenter (L.H. Engineering, Stoke Poges, Berkshire) in a medium containing 450 g brain heart infusion and 35 g neopeptone (both Difco). The inoculum was 50 ml of an overnight culture. Incubation was at 37 °C with gentle stirring (1·5 rev. min^{-1}) and automatic addition of 5 M-NaOH to maintain the pH at 7·2. After 16 h, sterile glucose was added to give a final concentration of 1 % (w/v), and incubation was then continued with stirring at 3·5 rev. min^{-1}. Growth was monitored spectrophotometrically at 600 nm and cultures were harvested at an E_{600} of 2·0 in a Sorvall continuous flow centrifuge at 4 °C.

Cultures (100 ml) were also grown in the synthetic medium of Tomasz (1964) using 0·5 % (w/v) casein hydrolysate instead of individual amino acids.

Preparation of crude enzyme. Harvested bacteria were washed with 0·7 % (w/v) NaCl in 10 mM-Tris/HCl buffer, pH 7·5, and disrupted with no. 11 Ballotini beads in a Braun homogenizer in 0·9 % NaCl. Walls and unbroken cells were removed by centrifuging at 6000 ***g*** for 10 min at 4 °C. The supernatant fluid was used as the crude enzyme.

Analytical methods. The protein content of cell-free extracts was determined by the method of Lowry *et al.* (1951). Choline phosphate and CDPcholine were detected on paper by the Dragendorf spray reagent (Bregoff, Roberts & Delwiche, 1953). High voltage electrophoresis was performed in a Shandon Southern HVE apparatus model L24 (Shandon Southern Instruments, Camberley, Surrey) on Whatman no. 1 or 3MM papers using the following buffers: buffer A, 8·0 % (w/v) formic acid, pH 1·8; buffer B, pyridine/acetic acid/water (5:5:190, by vol.), pH 4·7. Descending paper chromatography on Whatman no. 1 paper was carried out in solvent C: ethanol/0·5 M-ammonium acetate, pH 3·8 (15:6, v/v).

Assay of [methyl-^{3}H]choline phosphate. Reactions in incubation mixtures (see Results) were terminated by adding 3 vol. ethanol, mixing thoroughly and cooling in ice, and the insoluble material was removed by centrifugation. The supernatant fluid, which contained almost all of the ^{3}H, was dried by rotary evaporation in a small tube. The dried material was redissolved in 30 μl ethanol/water (1:2, v/v) and 10 μl samples were applied to Whatman no. 1 paper. Electrophoresis in buffer A was carried out for 30 min at 45 V cm^{-1}. Areas of the paper corresponding to standard choline phosphate were cut out and assayed for radioactivity in a scintillation fluid [containing 0·4 % (w/v) 2,5-diphenyloxazole (PPO) and 0·01 % (w/v) 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl POPOP) in toluene] using a Packard Tri-Carb model 3385 liquid scintillation spectrometer.

Assay of [methyl-^{3}H]CDPcholine. Incubation mixtures were treated as for the choline phosphate assay except, instead of electrophoresis, samples were chromatographed in solvent C and areas corresponding to standard CDPcholine were assayed for radioactivity.

RESULTS

Metabolites of [^{3}H]choline in whole bacteria

The defined medium of Tomasz (1964) (100 ml, containing 10 μCi [*methyl*-^{3}H]choline chloride) was inoculated with one loopful of *S. pneumoniae* and incubated statically for 16 h at 37 °C. The bacteria were then washed twice with ice-cold 0·9 % NaCl, once with distilled water and then extracted with 20 ml 5 % (w/v) trichloroacetic acid (TCA) for 30 min at 4 °C. The extract was separated by centrifuging at 10000 ***g*** for 15 min and the

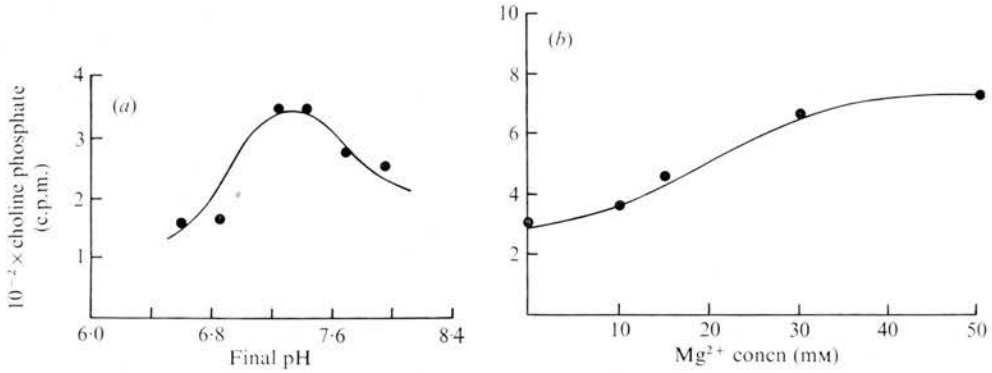

Fig. 1. (*a*) pH profile of choline kinase. Incubation mixtures (1·0 ml) containing 0·5 ml crude enzyme (8 mg protein), 0·2 ml 80 mM-veronal/HCl buffer, and (final concentrations) 0·04 % (w/v) 2-mercaptoethanol, 10 mM-Na_2HPO_4 and 10 mM-ATP, were incubated at 37 °C with 0·1 ml 40 mM-[*methyl*-^{3}H]choline chloride (0·5 μCi μmol^{-1}). After 20 min, duplicate 0·2 ml samples were assayed for choline phosphate and the pH value of the remainder was determined. (*b*) Magnesium ion dependence of choline kinase. $MgCl_2$ was added at (final concentrations) 0, 10, 15, 30 and 50 mM to 0·25 ml of an incubation mixture similar to that in (*a*). The whole incubation mixture was assayed for choline phosphate.

TCA was removed by six extractions with an equal volume of ether. Finally the extract was concentrated by rotary evaporation and chromatographed as a 2 × 0·5 cm band on Whatman no. 1 paper in solvent C. The distribution of radioactivity on the chromatogram was investigated by cutting the paper into 1 cm strips and measuring their radioactivity in a scintillation spectrometer. Results showed that 85 % of the radioactivity co-chromatographed with an authentic choline phosphate standard, 11 % co-chromatographed with CDPcholine and 4 % remained at the origin. No free choline was observed.

Cell-free studies on the enzymes of choline metabolism

Choline kinase (EC 2.7.1.32). In a preliminary experiment, choline, ATP and the crude enzyme were incubated together in a buffered system under conditions similar to those described in Fig. 1. Samples, taken at intervals over 60 min, showed a plateau for choline phosphate formation after about 30 min. This incubation mixture was then used as a basis for determining the optimum reaction conditions for the choline kinase. The enzyme had a pH optimum of between 7·3 and 7·4 (Fig. 1*a*), and its activity reached a maximum when incubated with about 30 mM-Mg^{2+} (Fig. 1*b*).

The kinetic properties of the pneumococcal choline kinase were examined for comparison with the corresponding yeast enzyme. Under the previously determined optimum conditions, the reaction choline → choline phosphate did not proceed any further (i.e. to the choline nucleotide). The enzyme's dependence on choline concentration was therefore investigated as follows: [*methyl*-^{3}H]choline chloride, at between 0 and 3 mM, was incubated with the crude enzyme suspension for 20 min at 37 °C with 3 mM-ATP. Choline phosphate was assayed and the enzyme activity was related to the substrate concentration by both direct and double reciprocal plots (Fig. 2*a*). A V_{max} of 0·5 nmol min^{-1} (mg protein)$^{-1}$ was obtained, with an apparent K_m of 0·19 mM. Using a constant choline concentration, and varying the ATP between 0 and 20 mM, the two plots of enzyme activity (Fig. 2*b*) showed that the substrate (ATP) strongly inhibited the enzyme at above 2·5 mM. Identical results were obtained with either 5 or 20 mM-Mg^{2+}, so the effect was not due to magnesium

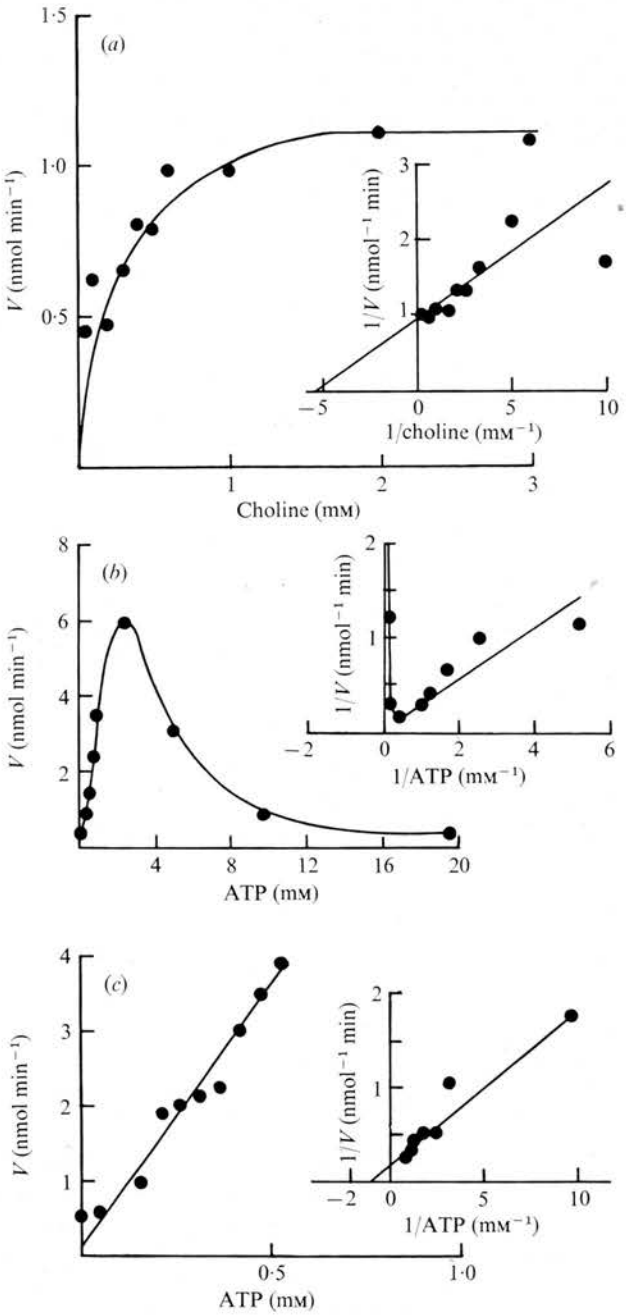

Fig. 2. Kinetics of pneumococcal choline kinase. Incubation mixtures (0·25 ml) contained 125 μl crude enzyme (2 mg protein) and (final concentrations) 40 mM-veronal/HCl buffer, pH 7·4, 0·01 % 2-mercaptoethanol, 2 mM-Na_2HPO_4 and 10 mM-$MgCl_2$. (*a*) [*methyl*-^{3}H]Choline chloride was varied between 0 and 3 mM with ATP constant at 3 mM. After 20 min at 37 °C, the whole mixture was assayed for choline phosphate. Results are plotted directly and as a double reciprocal plot (inset). (*b*) and (*c*) Similar incubation mixtures were used except the concentration of [*methyl*-^{3}H]-choline chloride was kept constant at 10 mM and ATP was varied between 0 and 20 mM (Fig. 2*b*) and 0 and 1 mM (Fig. 2*c*).

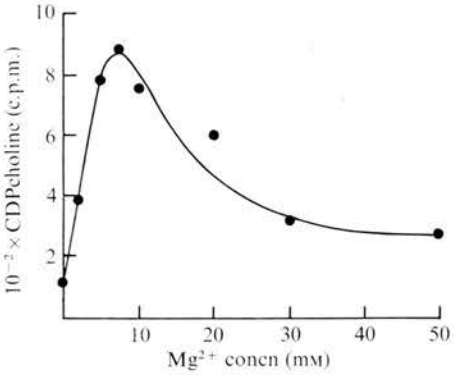

Fig. 3. Magnesium ion dependence of CDPcholine pyrophosphorylase. $MgCl_2$ was varied between 0 and 50 mM in incubation mixtures (0·25 ml) containing 60 μl crude enzyme, 120 μl 80 mM-veronal/HCl buffer, pH 8·4, and (final concentrations) 0·01 % 2-mercaptoethanol, 2 mM-Na_2HPO_4, 10 mM-CTP and 10 mM-[*methyl*-^{3}H]choline phosphate (0·06 μCi μmol^{-1}). After 20 min incubation at 37 °C, the whole incubation mixture was assayed for CDPcholine.

limitation. In a further experiment, in which ATP was varied between 0 and 1 mM, more accurate determinations gave V_{max} as 3 to 3·2 nmol min^{-1} (mg protein)$^{-1}$ and K_m as 1 mM (Fig. 2*c*).

Although no steps have been taken to purify the choline kinase in this study, we found that if the crude enzyme was centrifuged at 100000 *g* for 1 h, about 90 % of the enzyme activity per unit protein remained in the supernatant fluid.

CDPcholine pyrophosphorylase (EC 2.7.7.–). The experiment summarized above indicated that a choline nucleotide, with the same R_F as CDPcholine, was one of the metabolic products of choline. A preliminary experiment showed that CDPcholine could be synthesized in a cell-free system from choline phosphate and CTP: a mixture (1·0 ml) containing 0·5 ml crude enzyme, 20 mM-veronal/HCl buffer, pH 8·4, 0·01 % 2-mercaptoethanol, 2 mM-Na_2HPO_4, 10 mM-$MgCl_2$, 10 mM-[*methyl*-^{3}H]choline phosphate (0·06 μCi μmol^{-1}) and 10 mM-CTP was incubated for 50 min at 37 °C and 0·2 ml samples were taken at intervals and assayed for CDPcholine. The level of CDPcholine was observed to increase, reaching a plateau after about 30 min incubation.

CTP was a specific requirement of this enzyme. When it was replaced in the above incubation mixture by UTP, GTP or ATP at 10 mM and, after 30 min incubation, the whole mixture was assayed for choline nucleotides by chromatography in solvent C, no radioactivity was detected in the region between the origin and choline phosphate (where a choline nucleotide would be expected to run).

Using the incubation mixture described in Fig. 3 and varying Mg^{2+} between 0 and 50 mM, the maximum activity of the CDPcholine pyrophosphorylase was found with 7 mM-Mg^{2+} (Fig. 3).

No kinetic experiments were carried out on the CDPcholine pyrophosphorylase. Preliminary experiments on the crude enzyme system indicated that the reaction was not a simple one-step reaction. A variable amount of radioactivity, which could possibly be polymeric material, was always found at the origin. However, no free choline was found indicating the absence of a phosphatase.

DISCUSSION

The pneumococcus appears unique among bacteria in having choline in its cell wall. Choline is found widely in eukaryotic organisms as part of the phospholipid phosphatidylcholine (lecithin). This phospholipid is, however, relatively uncommon among bacteria (Hagen, Goldfine & Williams, 1966). Phosphatidylcholine is synthesized by two pathways in eukaryotes (Kennedy, 1962): (i) from CDP-D-1,2-diglyceride via phosphatidyl serine and phosphatidyl ethanolamine; or (ii) from choline via choline phosphate and CDPcholine plus D-1,2-diglyceride. There is evidence that both pathways occur in yeasts (Hunter & Rose, 1971; Brostrom & Browning, 1973), while the latter seems to be predominantly found in mammalian and plant systems. The metabolism of choline in the pneumococcus appears to be similar to the first two steps in pathway (ii). Tomasz (1967) has shown that ethanolamine cannot be methylated to choline by the pneumococcus.

The kinetics of the yeast choline kinase have been investigated by Brostrom & Browning (1973). The results obtained for the pneumococcal choline kinase, however, differ to some extent; the apparent Michaelis constant (K_m) with respect to choline was 190 μM for the pneumococcus and 15 μM for the yeast, whereas K_m with respect to ATP was 1·0 mM for the pneumococcus and 0·14 mM for the yeast. The pH optima also differ, that for the yeast being 8·5 to 9·5 compared with 7·3 to 7·4 for the pneumococcus. Both enzymes require magnesium. It should be noted that the yeast enzyme was partially purified and that K_m for the pneumococcal enzyme, especially with respect to ATP concentration, will be inaccurate as ATP will be metabolized in other ways. The assay procedure for the pneumococcal enzyme was simple and perhaps more reproducible than that used for the yeast enzyme.

Possibly the uptake of choline by *S. pneumoniae* involves the action of the kinase as there is no pool of free choline inside the cell. This could, however, be due to the bacteria being in the stationary phase of growth.

We propose that CDPcholine, which is synthesized from choline phosphate, is the direct precursor of the choline which is incorporated into pneumococcal C-teichoic acid. As not every repeating unit of C-teichoic acid is substituted with choline (I. R. Poxton & J. Baddiley, unpublished results), the control of choline substitution is probably at the level of the choline kinase or the CDPcholine pyrophosphorylase, possibly in combination with the phosphorylcholine esterase described by Holtje & Tomasz (1974).

In an independent study, Bean & Tomasz have shown that choline phosphate and CDPcholine are metabolic products of choline in the pneumococcus (B. Bean & A. Tomasz, unpublished results).

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The Uptake of Choline by *Streptococcus pneumoniae*

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Uptake of choline, a structural component of pneumococcal C- and F-teichoic acids, into bacteria growing in a defined medium was very efficient with an uptake constant ($[S]_{0.5}$) of 3·2 μM. It was inhibited by iodoacetate, dinitrophenol and oligomycin but not by structural analogues of choline. Ethanolamine, however, was transported in the absence of choline but with a reduced affinity ($[S]_{0.5}$ 71·4 μM). The same constitutive system was probably used by both ethanolamine and choline. It is suggested that this system required ATP and probably involved choline kinase.

INTRODUCTION

We recently demonstrated that choline, a nutritional requirement of *Streptococcus pneumoniae*, was metabolized into CDPcholine via choline phosphate by a cell-free extract of this organism (Poxton & Leak, 1977). This has also been shown independently by Bean & Tomasz (1977). Choline is utilized as a structural component of the wall teichoic acid or C-substance (Tomasz, 1967; Brundish & Baddiley, 1968) and of the membrane teichoic acid or F-antigen (Briles & Tomasz, 1973) of the pneumococcus.

We suggested that the first step in the metabolism of choline, i.e. its conversion to choline phosphate by the ATP-dependent choline kinase, may be involved in the transport mechanism for choline, as no pool of free choline was found in the cell. In the present study we have investigated this suggestion and attempt to suggest the mechanism whereby choline is taken up into the cell.

The uptake of ethanolamine, an analogue of choline, was also investigated. It is known that ethanolamine can replace choline in the pneumococcal cell envelope but that this alters some properties of the organism; for example, the daughter cells become unable to dissociate forming long chains, which are resistant to autolysis, detergent-induced lysis and infection by a bacteriophage, and also become unable to undergo genetic transformation (Tomasz *et al.*, 1975).

METHODS

Materials. [*methyl*-^{3}H]Choline chloride and [1-^{3}H]ethanolamine hydrochloride was purchased from The Radiochemical Centre, Amersham; the unlabelled compounds were from Sigma. Hemicholinium-3 was obtained from The Aldrich Chemical Co., Milwaukee, Wis., U.S.A.

Growth of organism. Streptococcus pneumoniae ATCC 12213 was periodically subcultured from freeze-dried stocks and maintained on blood agar plates at 4 °C for up to 14 d. It was grown statically in 25 ml Universal bottles at 37 °C with 20 ml filter-sterilized medium, similar to that used by Tomasz (1964) but

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containing 0·5 % (w/v) casein hydrolysate instead of individual amino acids. This medium contained choline at 28 μM. Cultures were routinely inoculated from plates into liquid medium, grown overnight at 37 °C, without shaking, then diluted into fresh medium to give an A_{600} of 0·05. After further incubation at 37 °C for approximately 1·5 h, A_{600} reached 0·1 (mid-exponential phase). At this density, cells were used for uptake experiments. Viable counts on blood agar plates indicated that $4{\cdot}5 \times 10^7$ colony-forming units (c.f.u.) ml^{-1} were present. If ethanolamine, at the same concentration, was used instead of choline, $9{\cdot}1 \times 10^6$ c.f.u. ml^{-1} were present at an A_{600} of 0·1. Total counts with a haemocytometer chamber showed that there were approximately twice as many individual cocci in ethanolamine medium as in choline medium for a given absorbance, with chains of, on average, about 30 cells in ethanolamine and 1 to 6 cells in choline.

Analytical methods. Descending paper chromatography was carried out on Whatman no. 1 paper using either ethanol/0·5 M-ammonium acetate, pH 3·8 (15:6, v/v) or ethanol/1·0 M-ammonium acetate, pH 7·5 (15:6, v/v) as the solvent. High voltage electrophoresis was performed on a Shandon electrophoresis apparatus (model L24) using Whatman no. 1 paper with either 8 % (v/v) formic acid (pH 1·8) or pyridine/acetic acid/water (5:5:190, by vol.; pH 4·7) as buffer.

Detection of compounds on paper. Choline and choline phosphate were detected by the Dragendorf spray reagent (Bregoff *et al.*, 1953). Ethanolamine was detected by ninhydrin and nucleotide derivatives by ultraviolet light.

Determination of incorporation of radioactive label. Radioactivity in choline phosphate and CDPcholine from cell-free extracts was determined as previously described (Poxton & Leak, 1977). To determine radioactivity in whole cells, 1 ml of the culture was filtered through a cellulose acetate membrane (13 mm diam., 0·45 μm pore size) held in a Swinnex holder, and the membrane was air-dried for 10 min at 37 °C. Instead of washing the membrane, which would introduce inconsistent results due to cell lysis, a background reading of medium containing the same concentration of label, without cells, was subtracted. Radioactivity was measured by counting the membranes in toluene scintillation fluid [8 g 2,5-diphenyloxazole and 200 mg 1,4-di-2-(4-methyl-5-phenyloxazolyl)benzene in 2 l toluene] using a liquid scintillation spectrometer.

Determination of $[S]_{0{\cdot}5}$ *values.* The value of $[S]_{0{\cdot}5}$ is the solute concentration giving one half of the maximum rate of uptake. This constant is preferred to the Michaelis–Menten constant for the measurement of uptake by whole cells as there are many metabolic controls on the transporting proteins in the system (Rose, 1976). Lineweaver–Burk graphs were constructed to calculate $[S]_{0{\cdot}5}$. In addition, linear regressions were calculated to obtain the equation of the straight line ($y = mx + c$) of the Lineweaver–Burk plots; $1/[S]_{0{\cdot}5}$ is the value of x when $y = 0$.

RESULTS AND DISCUSSION

When *S. pneumoniae* was grown overnight in a medium containing [*methyl*-^{3}H]choline chloride there was no free choline present in the intracellular pool of choline metabolites (Poxton & Leak, 1977). To confirm that no choline was free inside the bacterium, even in an actively growing culture with excess choline still present extracellularly, an overnight culture was inoculated into fresh medium with 28 μM-[^{3}H]choline chloride (5 μCi) to give an A_{600} of 0·05 and incubated statically until A_{600} reached 0·1 (about 1·5 h). The culture was then harvested and extracted with trichloroacetic acid as described by Poxton & Leak (1977). After removal of the trichloroacetic acid into ether, all of the ^{3}H was found in the aqueous phase. The chromatogram of the extract was similar to that obtained previously (except the amount of label at the origin, possibly due to polymeric teichoic acids, was much lower) with no trace of free choline. This confirmed that there was no free choline inside the cell.

In a similar experiment, bacteria were incubated in a medium containing 1 % (v/v) toluene to make the membrane permeable to choline and other small molecules up to a few thousand molecular weight (Schrader & Fan, 1974). Again there was no pool of choline in the cells and no stimulation in production of choline phosphate or CDPcholine.

Choline uptake by whole cells

Preliminary experiments to find the optimum specific activity of [^{3}H]choline to add to the incubations indicated that choline concentrations up to 1 μM containing 0·5 μCi $mmol^{-1}$ were suitable and the rate of uptake was linear over 30 min. Cells grown in the defined choline medium were harvested in mid-exponential phase (A_{600} 0·1) and resuspended in fresh medium without choline to give an A_{600} of about 0·05. After 30 min at 37 °C, when

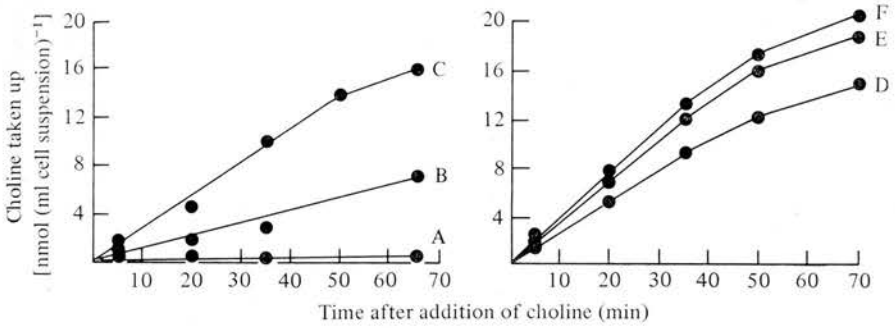

Fig. 1. Effect of inhibitors on the uptake of choline. Mid-exponential phase bacteria were suspended in synthetic medium containing [*methyl*-^{3}H]choline chloride (0·25 μM, 0·125 μCi ml^{-1}) and the following additions were made: A, 1 mM-iodoacetate; B, 1 mM-dinitrophenol; C, no addition; D, 0·25 mM-dinitrophenol; E, 0·25 mM-oligomycin; F, no addition. At intervals, 1 ml samples were taken and assayed for uptake of choline.

any choline intermediates would have been utilized, [^{3}H]choline was added up to 1 μM (0·5 μCi ml^{-1}). After a further 30 min the incorporation of choline into whole cells was measured. Results of uptake versus substrate concentration were plotted directly and as double reciprocal plots; these gave an $[S]_{0.5}$ value of 3·2 μM for choline and an uptake capacity 'V_{max}' of about 3 nmol choline min^{-1} (ml cell suspension)$^{-1}$.

Effect of choline analogues on choline uptake

Seven identical cultures, in choline-free medium as above, were preincubated for 30 min at 37 °C and then either hemicholinium-3, betaine hydrochloride, *N*,*N*-dimethylethanolamine, *N*-methylethanolamine, choline chloride (each at 5 μM) or an equivalent volume of choline- or analogue-free medium was added. Immediately after these additions, [^{3}H]choline chloride was added to give 0·5 μM (0·25 μCi ml^{-1}). After 30 min incubation at 37 °C, three 1 ml samples were assayed for incorporated ^{3}H. None of the analogues had inhibited the uptake of choline. Only with unlabelled choline was there a decrease of 50% due to dilution of the label.

These results indicate that there is a very specific transport mechanism for choline, with no inhibition when the analogues were present at 10 times the concentration of choline. This should be compared with the uptake of choline by a marine pseudomonad (apparently having no use for choline) which is inhibited by more than 50% in the presence of betaine hydrochloride and *N*,*N*-dimethylethanolamine (Snipes *et al.*, 1974).

Effect of metabolic inhibitors on the uptake of choline

Using mid-exponential phase cells harvested and suspended in choline-free medium as above, the uptake of [^{3}H]choline at 0·25 μM over 70 min was inhibited completely by iodoacetate (1 mM) and inhibited by 64% by dinitrophenol at 1 mM and 31% at 0·25 mM. Oligomycin (0·25 mM) inhibited uptake by only 12% (Fig. 1).

Iodoacetate is a general metabolic inhibitor which acts by alkylating thiol groups in a large number of enzymes. Oligomycin interferes with membrane ATPase and dinitrophenol reduces the proton-motive force across the cell membrane by destroying the proton gradient. The proton-motive force provides the energy for transporting certain sugars and amino acids and produces ATP in conjunction with membrane ATPase. The complete inhibition of choline uptake by iodoacetate and the comparative lack of sensitivity to dinitrophenol and oligomycin suggests that choline uptake is not dependent solely on the amount of ATP or the maintenance of the proton-motive force. We have, however, previously shown a requirement for ATP by the choline kinase (Poxton & Leak, 1977).

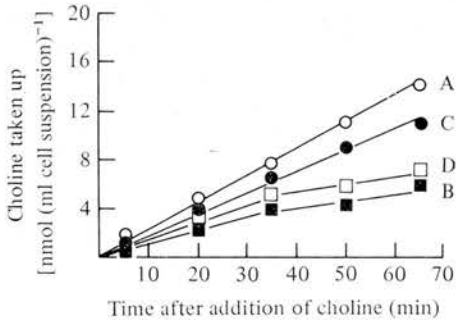

Fig. 2. Effect of chloramphenicol on the uptake of choline by choline-grown (A, B) and ethanolamine-grown (C, D) bacteria. Mid-exponential phase bacteria were suspended in medium free from choline and ethanolamine and 1 mM-chloramphenicol was added to tubes B and D. All tubes were incubated for 30 min before adding [*methyl*-^{3}H]choline chloride (0·25 μM, 0·125 μCi ml^{-1}). At intervals, 1 ml samples were taken and assayed for uptake of choline.

Ethanolamine uptake by whole cells

We investigated the uptake of ethanolamine as it led to altered properties of the organism when it replaced choline in the growth medium (see Introduction). *Streptococcus pneumoniae* was grown in medium containing ethanolamine (28 μM) overnight at 37 °C. The culture was diluted into fresh medium containing ethanolamine and harvested in mid-exponential phase. The kinetics of uptake were followed as for choline-grown organisms. The uptake constant was found to be much higher for ethanolamine than for choline ($[S]_{0·5}$ 7·14 μM) and the uptake capacity was correspondingly lower [V_{max} 4 nmol ethanolamine min^{-1} (ml cell suspension)$^{-1}$]. This suggests that the organism has a much greater affinity for choline than for ethanolamine.

Investigation of choline-induced transport system

Bacteria were cultured in both choline-based and ethanolamine-based media and harvested in mid-exponential phase. Each culture was then resuspended and preincubated in a choline- and ethanolamine-free medium with or without 1 mM-chloramphenicol. After 30 min, [^{3}H]choline (0·25 μM, 0·125 μCi ml^{-1}) was added and incubation was continued for 65 min at 37 °C. Throughout this period samples (1 ml) were assayed for choline uptake (Fig. 2).

There was no evidence from this experiment of induction of a choline transporting system in the ethanolamine-grown cells as the choline-grown control cells were similarly affected by chloramphenicol. The fact that chloramphenicol inhibited the uptake of choline suggests that protein synthesis is required to maintain choline uptake capacity.

As there was no evidence for induction of choline transport, and as we had earlier shown a difference in affinity for choline and ethanolamine when the organisms were grown on the homologous substrate, we investigated the change in uptake constants and capacities when the cells had been grown on one substrate and then changed over to the other substrate to follow uptake.

When the uptake of choline was assayed after bacteria had been grown on ethanolamine, $[S]_{0·5}$ was 1·4 μM-choline and V_{max} was 12 nmol choline min^{-1} (ml cell suspension)$^{-1}$. For the uptake of ethanolamine by choline-grown cells, $[S]_{0·5}$ was 14·2 μM-ethanolamine and V_{max} was 6 nmol ethanolamine min^{-1} (ml cell suspension)$^{-1}$. These results suggest that a maximum amount of enzyme is present in cells grown on ethanolamine thus giving an increased uptake capacity. When cells are grown on choline, however, a 'normal' amount of enzyme is present but this has a lower affinity for the uptake of ethanolamine.

Metabolism of ethanolamine

In an attempt to show the intermediates in ethanolamine metabolism, cells grown in [^{3}H]ethanolamine were extracted with trichloroacetic acid and treated as for choline-grown cells (Poxton & Leak, 1977). Regardless of incubation time and extraction procedure, we could not detect any intermediates. We therefore conclude that intermediates were being rapidly metabolized.

We conclude from this study that choline is transported into the pneumococcus by a highly specific constitutive enzyme which may be ATP-dependent. Choline kinase is probably involved. Choline analogues are transported by the same enzyme system but with a much lower affinity.

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Serological identification of *Bacteroides* species by an enzyme-linked immunosorbent assay

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Serological identification of *Bacteroides* species by an enzyme-linked immunosorbent assay

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SUMMARY An enzyme-linked immunosorbent assay was used to titrate antisera raised against live cultures of eight type species (biotypes) of *Bacteroides* with the EDTA-released outer-membrane complex from 29 characterised strains of *Bacteroides* species. With only minor exceptions, the strains investigated reacted to titre with the antisera raised against the homologous type species and not against the heterologous type species. Cross-reactivity between heterologous species and antiserum was only significant between closely related biotypes. This cross-reactivity could be removed by absorption of the antisera with whole cells. Significant correlation was found between serotype and biotype with this technique.

Most serological studies of *Bacteroides* have so far been limited to the sero-identification of selected species of *Bacteroides* by direct fluorescent antibody methods (Lambe and Jerris, 1976; Abshire *et al.*, 1977) and direct agglutination methods (Lambe and Moroz, 1976; Elhag *et al.*, 1977; Elhag and Tabaqchali, 1978a). These studies were concerned with the O-antigens of the strains in purified lipopolysaccharide (LPS) extracts or in preparations of heated or formalinised bacteria. Results suggest that there are a large number of serotypes within a given biotype (species or subspecies). The system is complex: the *B. fragilis* species alone includes 13 serotypes with different LPS antigens (Elhag and Tabaqchali, 1978a).

There is much interest in the possible use of serological techniques for the titration of serum antibodies to establish the involvement of a *Bacteroides* species in an infection, or to show the normal levels of antibodies directed against commensal strains of *Bacteroides* in healthy or compromised individuals. Several attempts have been made, mostly by isolating the organism from an established infection and using it as a source of the antigen (eg, Danielsson *et al.*, 1972), or by using type cultures of the Bacteroidaceae as sources of antigen (eg, Quick *et al.*, 1972; Rissing *et al.*, 1974). Many negative results were obtained from cases of proven infections involving identified *Bacteroides* species.

These studies all suffer from a basic lack of knowledge of the immunogenic structures of the *Bacteroides* cell. There is an apparent need for an antigen preparation that is both immunogenic and representative of a group of *Bacteroides* (either species or biotypes) rather than an individual strain. The disadvantages of the LPS molecule are that it is poorly immunogenic and that the O-antigen serotypes are so numerous within a given species or biotype (Hofstad, 1977; Elhag and Tabaqchali, 1978a). Kasper and co-workers have investigated the cell-surfaces of certain species of *Bacteroides* (Kasper and Seiler, 1975; Mansheim and Kasper, 1977) which appear to be typical Gram-negative organisms with an outer membrane and often a capsule. In a related study, Poxton and Brown (unpublished results) examined the outer membrane and associated proteins of several strains of *Bacteroides* by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and showed that similar biotypes (species) of *Bacteroides* possess many common polypeptides in their surface layers.

The present study exploits the group specific antigenicity of the cell-surface outer membrane complex together with the sensitivity of the enzyme-linked-immunosorbent-assay (ELISA) procedure to identify *Bacteroides* strains to species and subspecies level.

Material and methods

BACTERIA

Twenty-nine strains representing eight species of *Bacteroides* are listed in Table 1. They were obtained from culture collections or from laboratory stock

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Table 1 *Titration of homologous and heterologous antigens against representative antisera*

*Antigen prepared from**		*Titre of given antigen when titrated against antiserum raised against:*							
		NCTC9344	*NCTC10582*	*NCTC10583*	*ATCC8483*	*ATCC8503*	*VPI4196*	*NCTC9338*	*NCTC9337*
B. fragilis	NCTC9344	6400	3200	—†	—	—	—	—	—
	NCTC9343	6400							
	NCTC10584	6400							
	GNAB2A	6400	‡						
	GNAB4	6400							
	GNAB13	6400							
	WPH1	6400							
B. thetaiotaomicron	NCTC10582	400	6400	—	—	—	—	—	—
	GNAB1		6400						
	GNAB2		3200						
	GNAB11		3200	‡					
	GNAB20		3200						
B. vulgatus	NCTC10583	—	800	102400	—	—	—	—	—
	GNAB107			6400					
B. ovatus	ATCC8483	—	—	—	12800	—	—	—	—
B. distasonis	ATCC8503	—	1600	—	—	51200	1600	—	—
	GNAB26	—	1600			—			
B. melaninogenicus	VPI4196	—	6400	—	—	—	51200	6400	—
ss. *melaninogenicus*	ATCC15930						3200	800	—
	WPH62						6400	3200	—
	WPH67						25600	6400	—
	WPH97						25600	3200	—
B. melaninogenicus	NCTC9338	—	—	—	—	—	3200	25600	—
ss. *intermedius*	WPH4						6400	25600	—
	WPH22						3200	12800	—
	WPH24						3200	12800	—
	WPH31						3200	25600	—
B. asaccharolyticus	NCTC9337	—	800	—	—	—	—	—	51200
	WPH57						—	—	12800

*NCTC National Collection of Type Cultures.
ATCC American Type Culture Collection.
VPI Virginia Polytechnic Institute and State University, Blacksburg, Virginia.
GNAB and WPH Bacteriology Department, University of Edinburgh—Stock Cultures.
† = Titre of less than 400
‡Antigens from these strains were tested against homologous species antiserum only.

cultures (GNAB and WPH series) and characterised by the procedures of Duerden *et al.* (1976) and Holbrook *et al.* (1977).

GROWTH OF ORGANISMS AND PREPARATION OF ANTIGEN FOR ELISA

One millilitre of a 48-hour cooked meat broth culture (Watt, 1973) was used as an inoculum for 20 ml proteose peptone, yeast extract (PPY) medium (Holbrook *et al.*, 1977). After incubation at 37°C for 16 hours by the standard anaerobic procedure of Collee *et al.* (1972) the bacteria were harvested and washed twice in phosphate-buffered saline, PBS (0·15M NaCl containing 50 mM phosphate buffer pH 7·4) by centrifugation for 30 minutes at 4000 *g*. The pellets were resuspended in 2 ml PBS containing 10 mM ethylenediamine-tetraacetic acid (EDTA), incubated at 45°C for 30 minutes, and treated in an ultrasonic bath (Model 6441A, Dawe Instruments Ltd, Western Avenue, London W3 0SD, UK) for 60 seconds. The treated cells were removed by centrifugation at 6000 *g* for 30 minutes leaving the outer membranes and associated molecules in suspension. After dialysis for two successive periods of 2 hours in 2 litres of PBS this was used as the antigen preparation. The amount of protein in each antigen preparation was measured by the method of Lowry *et al.* (1951).

PREPARATION OF ANTISERA

Antisera were raised against whole live bacteria in New Zealand white rabbits which weighed about 2 kg. Five millilitres of blood were removed from each rabbit to provide control sera just before the first injection. Bacteria were cultured in 10 ml volumes of PPY medium, as described above, and washed three times in sterile PBS. They were finally resuspended in PBS to a concentration of 10^9 organisms/ml. The rabbits received 1 ml injections of this suspension intravenously according to the following schedule, with freshly cultured cells: weeks 1 and 2, daily injections for three days; week 3, no injection; week 4, one injection; week 5, test bleed (5 ml) then two or three days later the rabbits were exsanguinated by cardiac puncture. Sera were stored at −20°C (modified from Kasper, 1976).

ABSORPTION OF ANTISERA

Cross-reacting antibodies were absorbed from sera by incubating 1 ml of a 1 in 10 dilution (in PBS) of the serum with 1 ml of a suspension of washed bacteria prepared from 10 ml of an overnight culture in PPY medium, at 37°C for 30 minutes. After removing the bacteria by centrifugation (1000 *g*, 30 minutes) the serum was again added to an equal volume of washed bacteria and reincubated at 37°C for 30 minutes. The supernatant fluid obtained after centrifugation was equivalent to a 1 in 40 dilution of serum.

MICRO-ENZYME-LINKED IMMUNOSORBENT ASSAY

This was essentially the same as that described by Engvall and Perlmann (1972) but adapted for microtitre plates (flat-well plates supplied by Sterilin Ltd, Teddington, Middlesex, UK) as described by Voller *et al.* (1976). Fifty microlitres of antigen in 50 mM carbonate buffer pH 9·6, containing 0·02% sodium azide were added to each well and incubated at 37°C for 4 hours, then held at 4°C overnight. The plate was washed three times with 0·15M NaCl containing 0·05% Tween 20 by directing the nozzle of a wash-bottle into each well and filling it to the top. The plates were shaken dry between each wash. Antiserum, diluted in PBS containing 0·05% Tween 20 and 0·02% sodium azide, was added to each well (50 μl) and incubated at room temperature for 4 hours. The plates were washed as before and anti-rabbit IgG conjugated to alkaline phosphatase (Miles Laboratories (UK) Ltd, Stoke Poges, Slough, Bucks, UK), diluted in the same buffer as the antiserum, was added to each well (50 μl) and incubated overnight at room temperature. After again washing the plates, 50 μl volumes of the enzyme substrate—1 mg ml^{-1} solution of *p*-nitrophenylphosphate (Sigma) in 50 mM carbonate buffer, pH 9·8, containing 1 mM $MgCl_2$—were added to each well. After 1 hour's incubation at room temperature the colour was read visually on a $+++\rightarrow-$ scale. The end point of the titration was judged as $++$, that is, when the intense yellow colour had just started to decrease.

To determine the amount of outer membrane complex (antigen) required to coat the wells of a microtitre plate and to determine a suitable dilution at which the anti-rabbit IgG-alkaline phosphatase should be used, a chequer-board titration of doubling dilutions of antigen (1 mg protein/ml) against serial 1·5-fold dilutions of conjugate was carried out. An excess of homologous antiserum (1 in 100 dilution) was added to each well. A 1 in 16 dilution of antigen and a 1 in 500 dilution of conjugate were found to be suitable minimum values to give an optimum yellow colour and were the standard dilutions used for all subsequent experiments, each well being coated with antigen equivalent to *ca* 3 μg protein.

In trial experiments, antigens were titrated with homologous antisera, and titres between 6400 and and 102400 were obtained. To standardise conditions, all subsequent titrations started with an antiserum dilution of 1 in 400. At this dilution all preinoculation sera when titrated against antigen gave no trace of colour.

Results and discussion

TITRATION OF UNABSORBED SERA

Sera that had been raised against eight type species of *Bacteroides* were titrated with antigens prepared from both homologous and heterologous strains. The results are shown in Table 1. Significant cross-reactivity occurred between *B. fragilis* and *B. thetaiotaomicron*, *B. thetaiotaomicron* and *B. distasonis*, *B. melaninogenicus* subspecies *melaninogenicus* and *B. melaninogenicus* subspecies *intermedius*. A little cross-reactivity was observed between *B. melaninogenicus* subspecies *melaninogenicus* and *B. thetaiotaomicron*. It was possible to identify almost all of these strains to species or subspecies level without difficulty, the cross-reactive strains reaching an end point several dilutions below the titre of homologous strains and antiserum. All the strains of *B. fragilis* reacted to the same titre as the type species (NCTC 9344) against homologous antiserum. Similarly, most of the other species, where several strains were available for testing, reached titre with the antiserum raised against its type strain. Notable exceptions were *B. distasonis* (GNAB 26), which failed to react with its type strain antiserum but reacted significantly with *B. thetaiotaomicron* antiserum, and two strains of *B. melaninogenicus* ss. *melaninogenicus* (ATCC 15930 and WPH 62), which reacted only weakly with the antiserum raised against the type strain. In the latest VPI anaerobe manual (Holdeman *et al.*, 1977) it is stated that ATCC 15930 is an atypical *B. melaninogenicus* subspecies *melaninogenicus*. Another significant feature of these results is that the *B. asaccharolyticus* strains (previously *B. melaninogenicus* subspecies *asaccharolyticus*) do not cross-react at all with either of the *B. melaninogenicus* subspecies investigated and therefore appear to be unrelated serologically.

TITRATION OF ABSORBED SERA

It was possible to distinguish most of the species with unabsorbed sera, but there could be some confusion between certain *B. fragilis* and *B. thetaiotaomicron* strains and between the subspecies of *B. melaninogenicus*. An attempt was made to remove

Table 2 *Titration of antigens prepared from* B. melaninogenicus *subspecies* melaninogenicus *and subspecies* intermedius *with absorbed and unabsorbed sera raised against the type strains of these subspecies*

Antigen prepared from:		*Titre of given antigen when titrated against antiserum raised against:*			
		VPI 4196 Unabsorbed	*VPI 4196 Absorbed with NCTC 9338*	*NCTC 9338 Unabsorbed*	*NCTC 9338 Absorbed with VPI 4196*
B. melaninogenicus ss. *melaninogenicus*	VPI 4196	51200	51200	6400	—
	ATCC 15930	3200	400	800	—
	WPH 62	6400	400	3200	—
	WPH 67	25600	12800	6400	—
	WPH 97	25600	3200	3200	—
B. melaninogenicus ss. *intermedius*	NCTC9338	3200	—	25600	25600
	WPH 4	6400	800	25600	6400
	WPH 22	3200	—	12800	3200
	WPH 24	3200	—	12800	3200
	WPH 31	3200	—	25600	6400

— less than 400

cross-reacting antibodies from sera raised against these four strains. Thus NCTC 9344 antiserum was absorbed with NCTC 10582 cells and *vice versa*, while VPI 4196 serum was absorbed with NCTC 9338 cells and *vice versa*. Results showed that all cross-reactivity between *B. fragilis* and *B. thetaiotaomicron* could be removed by using absorbed sera, with no decrease in titre with homologous strains. Similarly, cross-reactivity between *B. melaninogenicus* ss. *melaninogenicus* and ss. *intermedius* could be removed by using absorbed sera (Table 2), but this was accompanied by a decrease in the titre of the absorbed sera when compared with the unabsorbed sera for some of the strains belonging to the same subspecies. This suggests a sharing of some major antigenic determinants between some members of both subspecies.

The species and subspecies investigated in this study have all been characterised by a variety of conventional biochemical tests and are therefore biotypes. Evidence presented here shows that similar biotypes also possess similar immunogenic surface components. As strains that belong to the same species or subspecies tend to react to titre, it should be possible to use the EDTA-released outer membrane complex as an antigen in a converse study to titrate serum antibodies, either in patients with a possible bacteroides infection or in normal or compromised individuals. If selected carefully and possibly pooled, a few antigen preparations could cover a very broad range of *Bacteroides* species. These should prove more suitable than the antigens used previously (see Introduction) which include LPS, and undefined heated, formalinised, or disrupted whole cells.

During the preparation of the manuscript Elhag and Tabaqchali (1978b) have published results of a survey of O-serotypes in the *B. fragilis* group (ie, *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus*, *B. ovatus* and *B. distasonis*). They found no correlation between O-serotype and biotype. This differs from the present finding that there is a close correlation between biotypes and serotypes as determined by reactions with antigens of the outer-membrane complex.

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Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis of Cell-surface Proteins as an Aid to the Identification of the *Bacteroides fragilis* Group

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The outer-membrane complexes from 40 strains of *Bacteroides*, representing eight of the species included in the *Bacteroides fragilis* group, were released by EDTA treatment. The component polypeptides were examined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis on slab gels. Within a species (biotype) the patterns produced indicated marked similarities in the structures of the surface proteins among the strains examined. The patterns produced by strains belonging to different species, however, showed fewer similarities. An unknown organism could therefore be identified to species level using this technique and a few selected biochemical tests.

INTRODUCTION

Bacteroides fragilis, *B. vulgatus*, *B. distasonis*, *B. thetaiotaomicron*, *B. eggerthii*, *B. ovatus*, *B. uniformis* and *B. splanchnicus* are species included in the *B. fragilis* group. They are characterized as being obligately anaerobic, non-pigmented, bile-tolerant, glucose-fermenting, Gram-negative rod-shaped bacteria. These organisms are frequently isolated from clinical specimens, predominantly from wounds, intra-abdominal infections, the blood and perirectal or pelvic sites (Finegold, 1977; Willis, 1977; Wren *et al.*, 1977). *Bacteroides fragilis*, a minor component of the faecal flora, is the member of the group most commonly isolated from infections, whereas *B. vulgatus*, *B. thetaiotaomicron* and *B. uniformis* are predominantly faecal isolates.

After an organism has been identified as a member of the family Bacteroidaceae by culture and microscopic examination, and is distinguished from the fusobacteria by gas–liquid chromatography of the volatile fatty acid metabolic products, at least eight, sometimes variable, biochemical tests have to be performed for identification to species level (Holdeman *et al.*, 1977).

Recently, polyacrylamide gel electrophoresis (PAGE) of cellular proteins has been employed in attempts to sub-classify the *Bacteroides* genus. Strom *et al.* (1976) used discontinuous gradient PAGE and examined proteins solubilized by sonication of whole cells of several species of Bacteroidaceae. Swindlehurst *et al.* (1977) used the SDS (sodium dodecyl sulphate)–PAGE procedure to examine members of the species *B. melaninogenicus*; these workers used hot SDS extracts of whole cells. It was possible to identify members of the Bacteroidaceae to species, subspecies and strain levels.

The present study evaluates the use of SDS–PAGE in examining the cell-surface proteins released from the bacteria by controlled treatment with EDTA at 45 °C followed by mild ultrasonic disruption. When this is used in conjunction with a few selected biochemical tests it becomes a relatively rapid, simple aid to identifying the species within the *B. fragilis* group.

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Table 1. *Strains of Bacteroides examined by SDS–PAGE and results of selected biochemical tests*

Species	Strain no.	Source	Indole production	Fermentation of: Rha	Tre	ManOH	Suc
B. fragilis	NCTC 9344	NCTC	−	−	−	−	NT
	NCTC 8560	NCTC	−	−	−	−	NT
	NCTC 10581	NCTC	−	−	−	−	NT
	NCTC 10584	NCTC	−	−	−	−	NT
	GNAB 4	Wound swab RIE	−	−	−	−	NT
	GNAB 8	Wound swab RIE	−	−	−	−	NT
	GNAB 49	Skin RIE	−	−	−	−	NT
	GNAB 101	Wound swab WGHE	−	−	−	−	NT
	GNAB 102	Wound swab WGHE	−	−	−	−	NT
	GNAB 104	Wound swab WGHE	−	−	−	−	NT
	GNAB 105	Wound swab WGHE	−	−	−	−	NT
B. vulgatus	NCTC 10583	NCTC	−	+	−	−	NT
	GNAB 25	Faeces	−	+	−	−	NT
	GNAB 30	Faeces	−	+	−	−	NT
	GNAB 31	Faeces	−	+	−	−	NT
	GNAB 34	Faeces	−	+	−	−	NT
	GNAB 35	Faeces	−	+	−	−	NT
	GNAB 37	Faeces	−	+	−	−	NT
	GNAB 38	Faeces	−	+	−	−	NT
	GNAB 44	Faeces	−	+	−	−	NT
	GNAB 107	Wound swab WGHE	−	+	−	−	NT
	GNAB 120	Wound swab WGHE	−	+	−	−	NT
B. distasonis	ATCC 8503	ATCC	−	+	+	−	NT
	GNAB 22	Faeces	−	+	+	−	NT
	GNAB 27	Faeces	−	+	+	−	NT
	GNAB 39	Faeces	−	+	+	−	NT
B. thetaiotaomicron	NCTC 10582	NCTC	+	+	+	−	NT
	GNAB 1	Wound swab RIE	+	+	+	−	NT
	GNAB 2	Wound swab RIE	+	+	+	−	NT
	GNAB 7	Wound swab RIE	+	+	+	−	NT
B. eggerthii	NCTC 11155	NCTC	+	+	−	−	−
	GNAB 21	Faeces	+	+	−	−	−
	GNAB 24	Faeces	+	+	−	−	−
	GNAB 41	Faeces	+	+	−	−	−
B. uniformis	ATCC 8492	ATCC	+	+	−	−	+
	VPI 11227	VPI	+	−	+	−	+
	GNAB 18	Wound swab RIE	+	+	−	−	+
	VPI 11368	VPI	+	+	−	−	+
B. ovatus	ATCC 8483	ATCC	−	+	+	+	+
B. splanchnicus	NCTC 10825	NCTC	+	−	−	−	NT

+, Positive; −, negative; NT, not tested.

Non-standard abbreviations: VPI, Virginia Polytechnic Institute, Blacksburg, Virginia, U.S.A.; RIE, Royal Infirmary, Edinburgh; WGHE, Western General Hospital, Edinburgh; GNAB, Lab. strain no.

Rha, Rhamnose; Tre, trehalose; ManOH, mannitol; Suc, sucrose.

METHODS

Growth of bacteria and preparation of protein extracts. Freeze-dried inocula of the strains listed in Table 1 were cultured in 10 ml of Robertson's cooked meat broth (modified by Watt, 1973) and incubated for 48 h at 37 °C by the standard anaerobic procedure of Collee *et al.* (1972). One ml of this culture was inoculated into 50 ml proteose peptone/yeast extract (PPY) medium (Holbrook *et al.*, 1977). After incubation for 20 h, 3 ml of the culture was removed for purity checks and biochemical testing (Table 1) while the remainder was harvested by centrifugation at 3000 *g* for 30 min and washed three times in phosphate buffered saline (0·15 M-NaCl in 50 mM-Na_2HPO_4/NaH_2PO_4 buffer, pH 7·4). The bacteria were resuspended in 2 ml phosphate buffered saline containing 10 mM-EDTA and transferred to a glass test tube. To remove the outer membranes

and associated molecules, the suspension was incubated for 30 min at 45 °C, then vortex-mixed, treated in an ultrasonic bath (model 6441A; Dawe Instruments, Western Ave, London) for 1 min and again vortex-mixed. After two centrifugations (6000 *g* for 30 min) the supernatant fluid contained outer-membrane vesicles together with released proteins and other molecules. The above conditions were standardized to give optimum release of protein without lysis of the bacterial cytoplasmic membrane. EDTA was removed by dialysis for 4 to 5 h in two successive 1 litre volumes of 0·01 M-Tris/HCl pH 7·4 containing 0·01 % (v/v) 2-mercaptoethanol and the samples were concentrated by dusting the dialysis bags with a fine layer of Sephadex G-100 and leaving at 4 °C for 16 h. The concentrated extracts were freeze-dried and dissolved in SDS–PAGE sample buffer to give a protein concentration of 2 mg ml^{-1}. Sample buffer was 0·0625 M-Tris/HCl pH 6·8 containing 2 % (w/v) SDS, 10 % (v/v) glycerol, 1 % (v/v) 2-mercaptoethanol and 0·001 % (w/v) bromophenol blue. Samples were heated in a boiling water bath for 3 min just prior to application to the gel.

Polyacrylamide gel electrophoresis. This was adapted from the method of Laemmli (1970). Slab gels (170 × 140 mm) of 10 % (w/v) acrylamide with a 10 mm 4 % (w/v) stacking gel were used to run up to 12 samples in a Raven (Haverhill, Suffolk, CB9 7UU) slab gel apparatus. Buffers were as described by Laemmli (1970). Samples (100 µl) of extract containing 200 µg protein were electrophoresed at constant voltage, first at 50 V until the sample had entered the separating gel (approx. 1 h) then at 150 V until the bromophenol blue was near the bottom of the gel (approx. 4 h). Staining was carried out overnight and de-staining was done over 4 h with the solutions described by Poxton & Sutherland (1976).

Protein estimation. Samples of EDTA extracts were assayed for protein using the method of Lowry *et al.* (1951).

Characterization of strains used. All test strains had previously been characterized by the procedures of Duerden *et al.* (1976). Selected biochemical tests, however, were performed on each strain after growth in PPY medium (Table 1). These served as a check on the previous results. They included tests on indole production and the fermentation of rhamnose, trehalose, sucrose and mannitol by the methods described by Duerden *et al.* (1976).

RESULTS AND DISCUSSION

The EDTA-extracted proteins from eight different type species included in the *B. fragilis* group were examined on the same gel (Fig. 1). The patterns of bands were quite different; only a few matching lines were shared and there was no possibility of picking out diagnostic bands typical of the *B. fragilis* group as a whole. However, the individual species within the group showed species-specific patterns.

For 11 strains of the species *B. fragilis* (syn. *B. fragilis* subsp. *fragilis*) there were many matching bands in all of the extracts (Fig. 2), perhaps with the exception of NCTC 8560 (track 2) which did not share many bands with any of the other strains. The double high molecular weight and triple low molecular weight sequences of bands (arrows) appeared to be common to the other 10 strains. There were close similarities between tracks 1 and 5 and between tracks 3, 7, 8, 9, 10 and 11.

Ten strains of *B. vulgatus* (syn. *B. fragilis* subsp. *vulgatus*) gave similar patterns with many matching bands (Fig. 3). The sequence of five or six high molecular weight bands (arrow) appeared to be characteristic of this species. Many other sequences of bands were shared by these strains and an overall similarity of patterns was observed.

Four strains of *B. distasonis* (syn. *B. fragilis* subsp. *distasonis*) were examined with a strain of debated identity (Fig. 4). All four strains of *B. distasonis* gave a strongly staining, high molecular weight band of similar, but not necessarily identical, molecular weight (arrow). The dubious strain (track 2, Fig. 4) had been received as *B. vulgatus* but the results of subsequent biochemical re-testing (Table 1) and the pattern produced on the gel confirmed that it was *B. distasonis*. It was, in fact, identical with strain ATCC 8503 (track 1, Fig. 4). Strains GNAB 27 and GNAB 39 (tracks 4 and 5, Fig. 4) showed almost identical patterns. These were faecal isolates taken several days apart from the same person.

Three indole-positive species of *Bacteroides* which were originally regarded as *B. thetaiotaomicron* (syn *B. fragilis* subsp. *thetaiotaomicron*) are now grouped into at least three separate species: *B. thetaiotaomicron*, *B. eggerthii* and *B. uniformis*. Their patterns on gels (Fig. 5) showed that they share several common polypeptides but there are closer similarities between strains from the same species. The four strains of *B. thetaiotaomicron* (tracks 1 to 4)

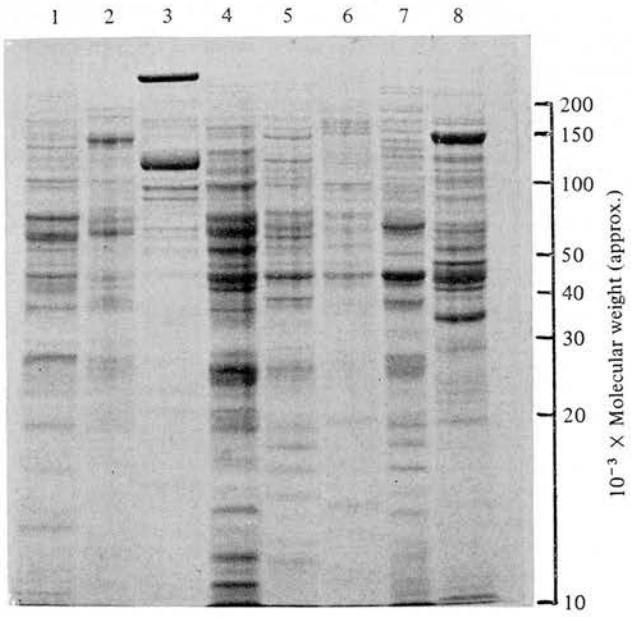

Fig. 1. SDS–PAGE of EDTA-extracted cell-surface proteins of *Bacteroides* species on 10% acrylamide slab gels (for details, see Methods). Track 1, *B. fragilis* NCTC 10584; 2, *B. vulgatus* NCTC 10583; 3, *B. distasonis* ATCC 8503; 4, *B. thetaiotaomicron* NCTC 10582; 5, *B. eggerthii* NCTC 11155; 6, *B. ovatus* ATCC 8483; 7, *B. uniformis* ATCC 8492; 8, *B. splanchnicus* NCTC 10825.

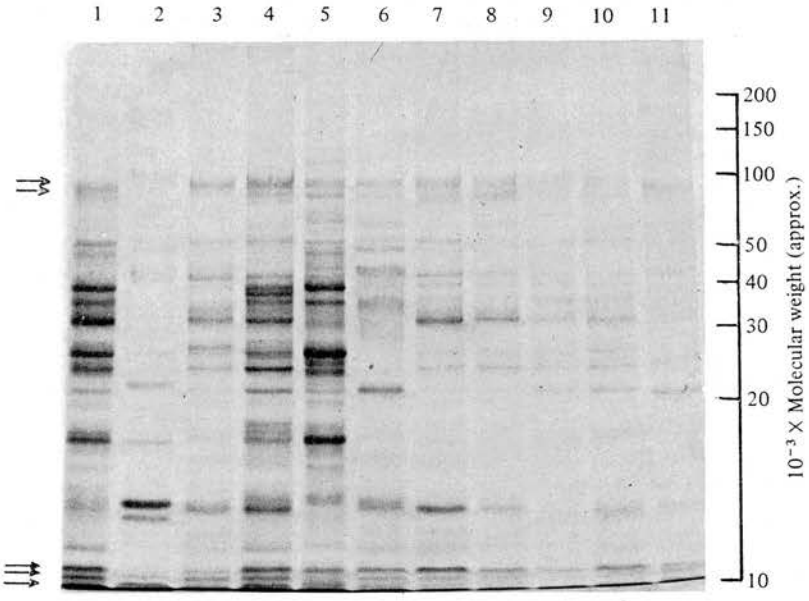

Fig. 2. SDS–PAGE of EDTA-extracted cell-surface proteins of strains of *Bacteroides fragilis* on 10% acrylamide slab gels (for details, see Methods). Track 1, NCTC 9344; 2, NCTC 8560; 3, NCTC 10581; 4, NCTC 10584; 5, GNAB 4; 6, GNAB 8; 7, GNAB 49; 8, GNAB 101; 9, GNAB 102; 10, GNAB 104; 11, GNAB 105. Arrows indicate bands characteristic of the species.

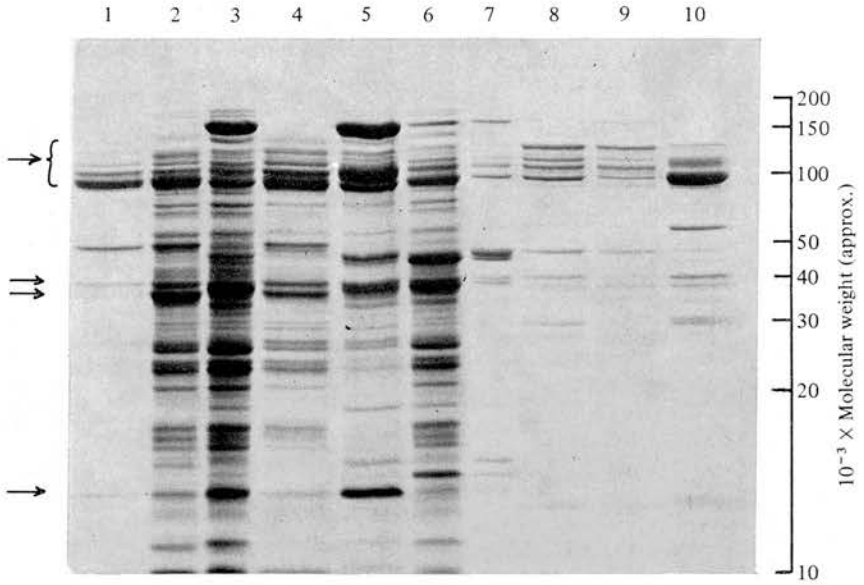

Fig. 3. SDS–PAGE of EDTA-extracted cell-surface proteins of strains of *Bacteroides vulgatus* on 10% acrylamide slab gels (for details, see Methods). Track 1, GNAB 25; 2, GNAB 30; 3, GNAB 31; 4, GNAB 34; 5, GNAB 35; 6, GNAB 37; 7, GNAB 38; 8, GNAB 44; 9, GNAB 107; 10, GNAB 120. Arrows indicate bands characteristic of the species.

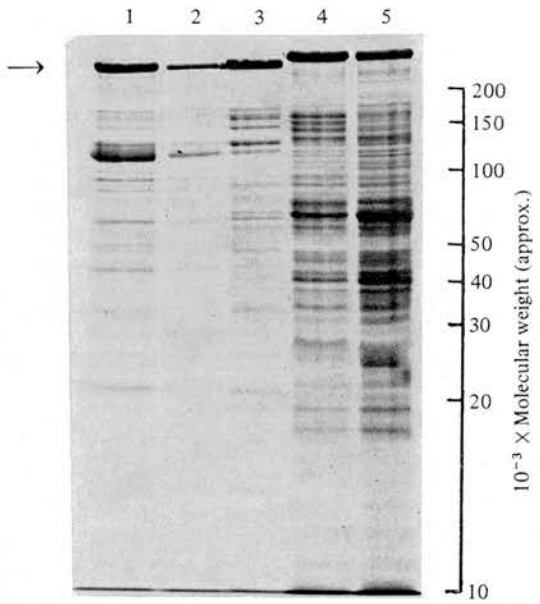

Fig. 4. SDS–PAGE of EDTA-extracted cell-surface proteins of strains of *Bacteroides distasonis* on 10% acrylamide slab gels (for details, see Methods). Track 1, ATCC 8503; 2, mis-labelled *B. vulgatus*; 3, GNAB 22; 4, GNAB 27; 5, GNAB 39. Arrow indicates a band characteristic of the species.

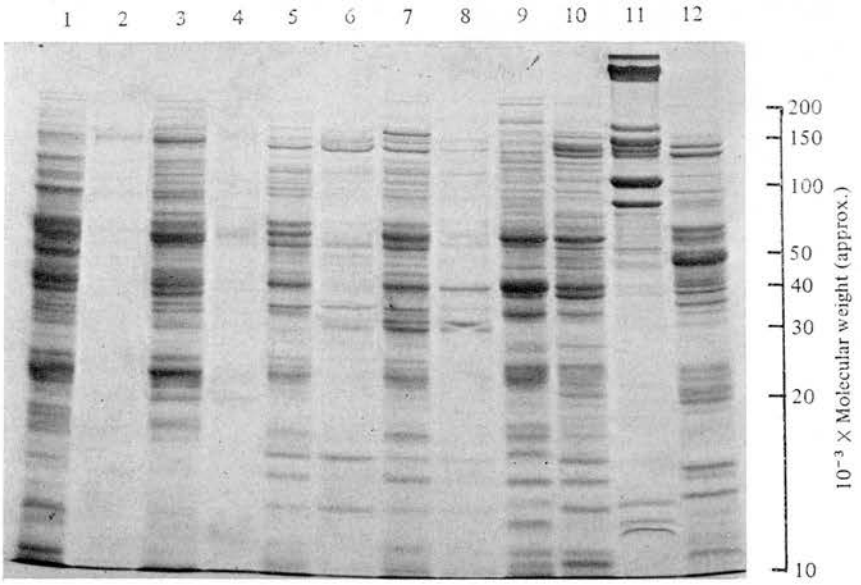

Fig. 5. SDS–PAGE of EDTA-extracted cell-surface proteins of strains of indole-positive *Bacteroides* species on 10 % acrylamide slab gels (for details, see Methods). *Bacteroides thetaiotaomicron*: track 1, NCTC 10582; 2, GNAB 1; 3, GNAB 2; 4, GNAB 7. *Bacteroides eggerthii*: track 5, NCTC 11155; 6, GNAB 21; 7, GNAB 24; 8, GNAB 41. *Bacteroides uniformis*: track 9, ATCC 8492; 10, VPI 11227; 11, GNAB 18; 12, VPI 11368.

showed many matching bands. Similarly, the four strains of *B. eggerthii* (tracks 5 to 8) closely matched each other. The strains of *B. uniformis* (tracks 9 to 12), however, showed variation: strains ATCC 8492 (track 9) and VPI 11227 (track 10) matched very closely, and strain VPI 11368 (track 12) shared several common polypeptides with these two; however, strain GNAB 18 (track 11) was very different from these although the results from standard biochemical tests showed it to be identical with VPI 11368. Tracks 2 and 4 were unfortunately weakly stained although the amount of protein was the same in all the samples applied to this gel. A duplicate gel, using freshly prepared samples, gave the same result. In the original gel the bands in tracks 2 and 4 could be easily compared with 1 and 3.

This study presents much evidence that similar biotypes of *Bacteroides* possess similar polypeptides in their surface layers. The patterns of *B. fragilis* NCTC 8560 and *B. uniformis* GNAB 18 show, however, that organisms indistinguishable by biochemical tests can differ significantly in their surface chemistry; thus the results of biochemical tests alone can be misleading and should be used with caution. Due to our present lack of knowledge of the cell-surface chemistry and serology of *Bacteroides* we are not in a position to re-classify strain GNAB 18.

The characterization of the *Bacteroides* genus by conventional biochemical techniques is time-consuming and some of the tests are unreliable. SDS–PAGE of surface proteins in conjunction with a few selected biochemical tests offers a rapid, reproducible approach to the demonstration of relatedness between an unknown and a type species. It is hoped that the fine degree of accuracy of this analysis will make it possible to distinguish small differences between strains, so that it might even be possible to distinguish between a newly acquired organism and a resident strain in a patient.

This work was supported by the Medical Research Council (grant no. G977/951/S). We are grateful to Professor J. G. Collee for his interest and helpful advice, to Professor B. P. Marmion who initially suggested the approach and to Mr G. Hay and Miss M. D. Byrne for skilled technical assistance.

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Characterization of the surface immunogens of bacteroides and their use in sero-identification

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Until recently the characterization of bacteroides organisms to species level depended almost exclusively on conventional biochemical tests and simple microscopic observations. There have been difficulties in the development or acceptance of serological tests. Those based on lipopolysaccharide antigens appear to be of little use in species identification as the O-serotypes are numerous and not species-specific[1]. Recent reports, however, suggest that some capsular antigens may be species-specific and useful in sero-identification of some *Bacteroides* species[2,3].

Bacteroides organisms appear to be morphologically and chemically typical Gram-negative bacteria. Our preliminary analyses of the cell-surface (outer-membrane) proteins of a number of strains of different species of bacteroides by sodium dodecyl sulphate-polyacrylamide gel electrophoresis have shown that the patterns of peptides were very similar within a species but were significantly different between species[5]. This suggested that it might be possible to develop a sero-identification system based on cell-surface protein antigens. An indirect enzyme-linked immunosorbent assay (ELISA) has been used for this investigation[4]. Antisera were raised against live, untreated organisms. The antigens used for coating the polystyrene wells of microtitre plates prepared by treatment of the bacteria with EDTA, heat and mild ultrasonic shearing. When the antisera were titrated in ELISA tests against these antigens, species-specific reactions were clearly obtained (Tables I and II). Cross-reactions could be removed by absorption of the sera with whole bacteria.

At present this ELISA procedure has made it possible to use type-specific antisera to identify *B. fragilis*, *B. thetaiotaomicron*, *B. eggerthii*, *B. vulgatus*, *B. melaninogenicus ss. melaninogenicus* and *ss. intermedius*, *B. asaccharolyticus*, *B. bivius* and *B. disiens*. Other species of bacteroides are more complex and it appears that some, such as *B. distasonis*, might contain at least two serogroups within the species.

In order to define the numbers and types of immunogenic molecules from the cell surface reacting in the ELISA procedure, characterization of the immunogens was attempted. This has been achieved by the three methods outlined below. The ELISA procedure was used as the test system for the first two methods which exploited the ability of a variety of agents to denature or modify the antigens.

1. The antigens to be used for coating the polystyrene wells were treated with the agents specified in Table III and the changes in titre were observed. The results shown in Table III indicate that the denaturing agents had different effects on the two species investigated. The findings suggest that the major immunogenic molecules of *B. vulgatus* are protein, whereas those of *B. melaninogenicus ss. melaninogenicus* include both protein and carbohydrate.

Table I

Antigen prepared from:		Titre when antigen was titrated with antiserum raised against:			
		B. fragilis NCTC 9344	*B. th. i. o.* NCTC 10582	*B. eggerthii* NCTC 11155	*B. vulgatus* NCTC
B. fragilis	NCTC 9344	6400	3200	—	—
	NCTC 9343	6400			
	NCTC 10584	6400			
	NCTC 10581	6400			
	NCTC 8560	6400			
	GNAB 4	6400			
	GNAB 13	6400			
	WPH 1	6400			
B. thetaiotaomicron	NCTC 10582	400	6400	—	—
	GNAB 1		6400		
	GNAB 2		3200		
	GNAB 7		3200		
	GNAB 11		3200		
	GNAB 20		3200		
B. eggerthii	NCTC 11155			6400	
	GNAB 21			3200	
	GNAB 24			3200	
	GNAB 41			3200	
	GNAB 43			3200	
B. vulgatus	NCTC 10583	—	800	—	102400
	NCTC 11154				102400
	GNAB 9				25600
	GNAB 25				51200
	GNAB 30				51200
	GNAB 107				6400
	GNAB 120				51200
	WPH 116				25600

— Less than 400

2. To confirm that the change in titre was due to the inactivation of the antigens, and not the result of a non-specific effect such as inhibition of the binding of the antigen to the polystyrene wells, an ELISA-inhibition test was used. The wells were coated as usual with whole outer membrane complex, but before addition of the antiserum (at a dilution equivalent to half of its titre) the antiserum was preincubated with various antigen preparations. A 100% inhibition (positive control) was achieved by preincubating the serum with whole outer membrane complex. Results are summarized in Table IV. This confirmed that the major immunogens of *B. vulgatus* are protein whereas carbohydrate antigens other than lipopolysaccharide and capsular polysaccharide seem to be significant in *B. melaninogenicus ss. melaninogenicus*.
3. A crossed immunoelectrophoresis (CIE) procedure has also been developed to assist quantitation and characterization of the immunogenic molecules of *Bacteroides spp*. Results so far show that in each of a few species investigated there are three or more major antigens on the cell surface together with several minor antigens.

With ELISA and CIE procedures it should be possible to define the immunogens of the cell surface of bacteroides organisms. These findings are promising in relation to sero-identification within the genus and determination of antigenic molecules that may be shared by closely related species or subspecies.

Table II

Antigen prepared from:		Titre when antigen was titrated with antiserum raised against:		
		B. mel. mel. VP1 4196	*B. mel. intermed.* NCTC 9338	*B. asaccharolyticus* NCTC 9337
B. melaninogenicus ss. melaninogenicus	VP1 4196	51200	6400	—
	ATCC 15930	3200	800	
	WPH 62	6400	3200	
	WPH 67	25600	6400	
	WPH 97	25600	3200	
B. melaninogenicus ss. intermedius	NCTC 9338	3200	25600	—
	WPH 4	6400	25600	
	WPH 22	3200	12800	
	WPH 24	3200	12800	
	WPH 31	3200	25600	
B. asaccharolyticus	NCTC 9337	—	—	51200
	B 2296			25600
	B 3502			12800
	B 3586			12800
	WPH 57			12800

— Less than 400

Table III Effect of pre-treatment of coating antigen on titre of homologous antiserum

Treatment of antigen	Titre of homologous serum with antigen from	
	B. vulgatus (NCTC 10853)	*B. mel. ss. mel.* (VPI 4196)
None	12800	25600
0.1M $NaIO_4$ (overnight)	12800	400
20% HCHO (overnight)	3200	6400
121°C 15 min.	400	6400

Table IV Inhibition of ELISA

Inhibitor	Inhibition (%)	
	B. vulgatus	*B. mel. ss. mel.*
Outer membrane (OM)*	100	100
Heat-treated OM	0	0
Urea-treated OM	0	ND
Formalin-treated OM	50	50
Periodate-treated OM	100	0
LPS	tr	0
Capsule	ND	0
None†	0	0

*Positive control
†Negative control
tr = trace
ND = not determined

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The Cell-surface Antigens of *Bacteroides vulgatus*

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The cell surface of *Bacteroides vulgatus* was examined by electron microscopy. The outer membrane complex was removed by EDTA and mild sonication and the antigens of this complex were characterized by enzyme-linked immunosorbent assay and crossed immunoelectrophoresis. The species-specific antigen was identified and was shown to be the major outer membrane protein with a molecular weight of 100000.

INTRODUCTION

Bacteroides vulgatus, an obligately anaerobic, Gram-negative, rod-shaped bacterium is the predominant bacteroides organism in the human colon (Finegold *et al.*, 1975; Duerden, 1980). It is occasionally isolated from clinical specimens (Holland *et al.*, 1977). Recently, it was shown that the sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) profiles of the outer membrane (OM) polypeptides of several *B. vulgatus* strains were extremely similar (Poxton & Brown, 1979); and the antigens of the OM complex of bacteroides appear to be species- or subspecies-specific when detected by an enzyme-linked immunosorbent assay (Poxton, 1979).

It is becoming recognized that proteins of the OM of other Gram-negative bacteria may include group- and species-specific antigens that might be useful in serological identification (e.g. Hofstra & Dankert, 1980; Sippel *et al.*, 1978).

The present study characterizes the antigens of the OM complex of *B. vulgatus* and investigates the species-specific antigens. The recognition of species-specific antigens of bacteroides would greatly aid the development of more rapid methods for their identification.

METHODS

Culture of bacteria and preparation of EDTA–outer membrane complex. Bacteroides vulgatus NCTC 10583 and NCTC 11154, and six laboratory strains (GNAB 9, 25, 30, 107, 120 and WPH 116) which had been characterized in detail according to the criteria of Duerden *et al.* (1980), were cultured anaerobically from freeze-dried inocula in PPY medium (Holbrook *et al.*, 1977) and outer membrane (OM) complexes were prepared by the EDTA–heat–mild sonication treatment previously described (Poxton & Brown, 1979).

Electron microscopy. This was based on the method of Springer & Roth (1973). Bacteria were harvested from blood agar plates (5% human blood in Columbia base; Oxoid) or from PPY medium, and suspended in a mixture of 1 ml ruthenium red (1·5 mg ml^{-1}) in water, 1 ml 3·6% (w/v) glutaraldehyde and 1 ml 0·2 M-sodium cacodylate buffer pH 6·5 and held at 0 °C for 1 h. After three washes in 0·07 M-cacodylate buffer, the pellet was suspended in a mixture of 1 ml ruthenium red (1·5 mg ml^{-1}), 1 ml 4% (w/v) osmium tetroxide and 1 ml 0·2 M-cacodylate buffer and incubated for 3 h at 27 °C. The pellet was washed once in 0·07 M-cacodylate buffer and dehydrated in successive 10 min steps in 25%, 50%, 75% and 90% (v/v) ethanol, then in absolute ethanol for two periods of 1 h. Propylene oxide was added for two 10 min periods and the pellet was then kept in a propylene oxide/Epon Araldite (1:1, v/v) mixture. The pellet was embedded in fresh Epon Araldite in disposable capsules and maintained at 45 °C for 24 h then at 60 °C for 48 h. Sections were cut and placed on rhodium-coated copper grids. The thin sections were stained with saturated uranyl acetate in 75% ethanol in the dark for 30 min, then washed once in

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75% ethanol and once in distilled water and stained for 2 min with Reynolds' lead citrate (Reynolds, 1963). They were then washed once in 0·02 M-NaOH and three times in water, blotted dry, and viewed in a Hitachi Model HU 12A electron microscope.

Thin sections of the OM vesicles were prepared as above except that they were initially pelleted at 100000 ***g*** for 1 h and ruthenium red was omitted from the fixation procedure.

Capsule preparation. Blood agar plates were flood-seeded with a 16 h PPY culture of *B. vulgatus* NCTC 10583. To enhance capsule production, this strain had been passaged for two 6 h periods in the peritoneum of a mouse. After 72 h anaerobic incubation at 37 °C the cells were harvested by scraping, with a glass slide covered with adhesive tape, into 0·15 M-NaCl in 0·05 M-phosphate buffer pH 7·4 (PBS) containing 0·08% (v/v) formaldehyde. The suspension was mixed vigorously in a Waring blender for 30 s. Bacteria were removed by centrifugation (6000 ***g***, 30 min) leaving the capsular material in solution. This was precipitated by the addition of 4 vol. acetone at −18 °C. The precipitate was recovered by centrifugation (4000 ***g***, 30 min), washed once in acetone, dissolved in water and dialysed against distilled water. To purify the carbohydrate fraction from the crude capsular material it was extracted with aqueous phenol: an equal volume of 90% (w/w) phenol was added to the solution of capsular material and stirred vigorously for 15 min at 20°C. The phases were separated by centrifugation (6000 ***g***, 30 min) and the upper phase was dialysed for 16 h against running tap water, then against distilled water, and finally freeze-dried.

Lipopolysaccharide (LPS) preparation. LPS was extracted from freeze-dried bacteria that had been grown for 18 h in PPY medium, by the aqueous phenol procedure developed by Westphal & Lüderitz (1954). It was purified and washed by centrifugation at 100000 ***g*** for 3 h.

Treatments of OM complex. Separate samples of the EDTA-released OM complex at a concentration of 0·5–1·0 mg protein ml^{-1} were subjected to one of the following denaturing or modifying agents: (i) heat (121 °C for 15 min); (ii) formaldehyde (20% at 20 °C for 16 h); (iii) sodium periodate (0·1 M at 20 °C for 16 h; excess periodate was consumed by the addition of ethylene glycol); (iv) urea (8 M at 20 °C for 16 h); (v) pronase (0·1 mg ml^{-1} in PBS at 20 °C for 16 h); (vi) trypsin (as for pronase). Excess formaldehyde, urea and the periodate reagents were removed by dialysis against PBS and enzymes were inactivated by the addition of formaldehyde to a final concentration of 0·2%. Controls were included, in which the OM samples were treated as above except that the agent was omitted.

Antiserum. Antiserum was raised against washed live cells of *B. vulgatus* NCTC 10583 in New Zealand White rabbits as previously described (Poxton, 1979).

Enzyme-linked immunosorbent assay (ELISA). Both the indirect ELISA described by Poxton (1979) and an ELISA-inhibition test were used. The ELISA-inhibition was performed as for the indirect test, except that the antiserum was pre-incubated with the potential inhibitor: antiserum (50 µl), diluted to a concentration twice that of its titre, was incubated with an equal volume of doubling dilutions of treated OM complex (see above) or LPS (1 mg ml^{-1}) for 30 min in a 37 °C water bath; 50 µl volumes of this pre-incubated serum were added to each well. Inhibition was observed by comparing the test with a positive and negative control.

SDS–PAGE. This was performed on 10% (w/v) acrylamide slab gels, with the buffer system of Laemmli (1970), by the method described by Poxton & Brown (1979).

Crossed immunoelectrophoresis (CIE). EDTA–OM complexes and isolated LPS and capsular polysaccharide were examined by CIE according to the method developed by Weeke (1973), as described by Poxton & Byrne (1981). CIE was also performed, as indicated, with SDS–PAGE in the first dimension.

Analytical techniques. Protein concentrations were estimated by the Lowry method, and carbohydrate as glucose equivalents was estimated by the method of Dubois *et al.* (1956).

RESULTS AND DISCUSSION

Electron microscopy of thin sections of *B. vulgatus* NCTC 10583 demonstrated the typical appearance of the cell envelope of a Gram-negative bacterium (Fig. 1). The inner or cytoplasmic membrane and the outer membrane were clearly visible, and separated by a dense layer corresponding to peptidoglycan. On the outer surface of the outer membrane there was a thin capsule layer. Electron microscopy of the bacteria after the EDTA–heat–sonication treatment did not reveal any dramatic differences from the untreated organisms, except that the capsular layer was absent. There were no obvious examples of organisms with part of the outer membrane removed as shown for *B. fragilis* by Kasper & Seiler (1975). When the material released by EDTA was examined in the electron microscope, after sedimenting at 100000 ***g*** for 1 h, vesicles of varying size bounded by a

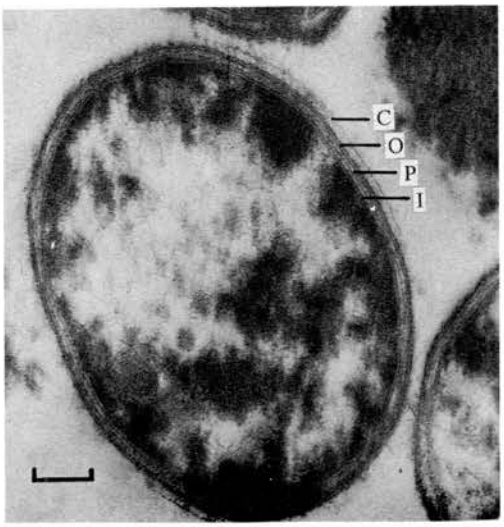

Fig. 1

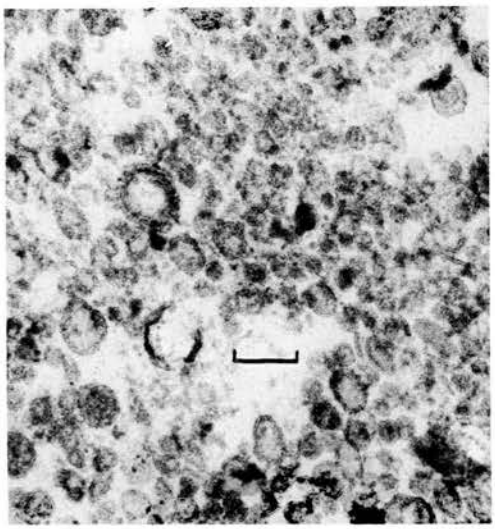

Fig. 2

Fig. 1. Thin section of *B. vulgatus* NCTC 10583 stained with ruthenium red. Inner (I) and outer (O) membrane, peptidoglycan (P) and capsule (C) are visible. The bar marker represents 0·1 μm.

Fig. 2. Thin section of EDTA-released outer membrane vesicles which were deposited by ultracentrifugation at 100000 ***g*** for 1 h. The bar marker represents 0·1 μm.

single trilamellate membrane were observed (Fig. 2). This suggests that after fragments of outer membrane had been removed the outer layer of the organism was re-formed.

Chemical analysis of the EDTA-released OM complex showed it to be predominantly protein with a small proportion of carbohydrate (protein : carbohydrate, approximately 14 : 1 by weight). SDS–PAGE revealed at least 30 polypeptides, with two major bands at 100000 and 70000 molecular weight. After ultracentrifugation (100000 ***g***, 1 h), the pellet was very similar to the whole EDTA-released complex except that the polypeptide of 70000 molecular weight was predominantly in the supernate (Fig. 3).

When the EDTA-released OM complex from eight strains of *B. vulgatus* was titrated with antiserum raised against *B. vulgatus* NCTC 10583 by the indirect ELISA technique, titres of 25600–102400 were obtained, whereas titres of less than 400 were obtained when EDTA antigens from 17 other species of bacteroides were titrated with the *B. vulgatus* antiserum.

The homologous antigen–antibody reaction was visualized by crossed immunoelectrophoresis (CIE). At least seven precipitin lines, including line 3, were visible (Fig. 4). When this CIE profile was compared with a CIE profile of the ultracentrifuged pellet, only lines 2 and 5 appeared not to be membrane bound. When antigens from the other seven strains, which strongly react with the NCTC 10583 antiserum in ELISA, were reacted with this antiserum in CIE, line 3 was produced by all; in addition, antigens prepared from two of them (NCTC 11154 and GNAB 120) produced line 7. No other precipitin lines, either anodic or cathodic, were produced by these seven strains. This indicates that the cross-reaction demonstrated by ELISA is due to one major species-specific antigen associated with precipitin line 3 in CIE.

Several approaches were made to determine the identity of the antigens of the OM complex of strain NCTC 10583 and the major species-specific antigen which forms precipitin line 3 in CIE. Initially, a crude screening determination was made by ELISA-inhibition (see Methods). Complete inactivation of the antigens could only be demonstrated by heating the OM complex for 15 min at 121 °C. Treatment with 8 M-urea resulted in almost complete

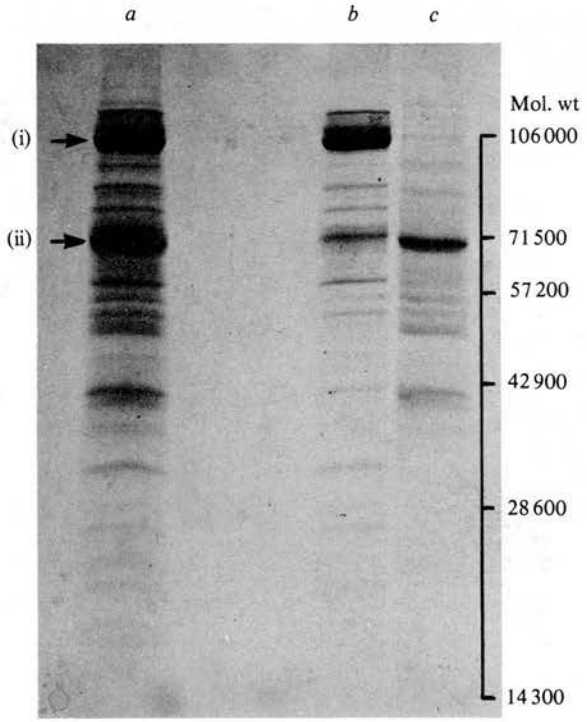

Fig. 3. SDS–PAGE of EDTA-released outer membranes of *B. vulgatus* NCTC 10583 on 10% acrylamide gels: (*a*) whole EDTA extract; (*b*) ultracentrifuged pellet (100000 *g*, 1 h); (*c*) ultracentrifuge supernate. The arrows indicate the major membrane-bound (i) and unbound (ii) polypeptides.

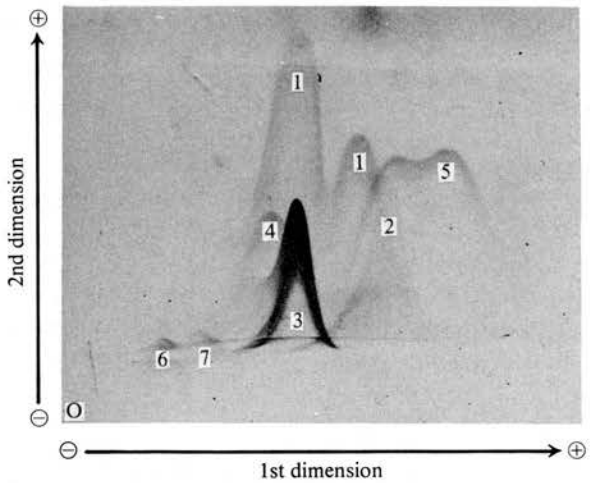

Fig. 4. Crossed immunoelectrophoresis of EDTA-released OM complex (20 μg protein) from *B. vulgatus* NCTC 10583 against homologous whole cell antiserum (0·25 ml in 3·5 ml agarose) on 50 × 50 mm glass slides. Electrophoresis was at 12·5 V cm^{-1} for 1·5 h in the first dimension and 12 V cm^{-1} for 16 h in the second dimension, both at 4 °C. Note that precipitin lines 1 and 5 each form a double peak. O, origin.

inactivation, whereas formaldehyde, trypsin, pronase and periodate produced only slight or partial inactivation. Inhibition by isolated LPS was minimal under circumstances that we cannot claim to be quantitative.

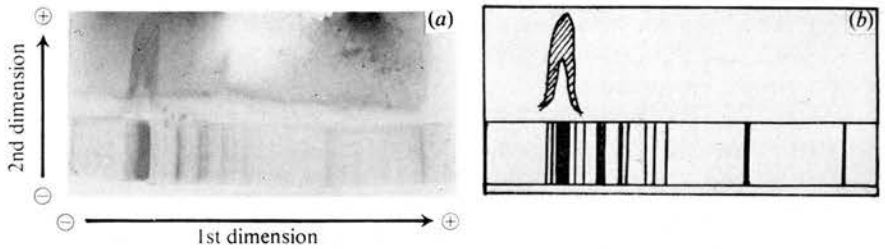

Fig. 5. SDS–PAGE/crossed immunoelectrophoresis of OM complex (50 μg protein) from *B. vulgatus* GNAB 25. Outer membrane complex, after SDS–PAGE on a 10% acrylamide gel in the first dimension, was run into 3·5 ml 1% (w/v) agarose containing 0·25 ml whole cell antiserum of *B. vulgatus* NCTC 10583 and 1% (w/v) Triton X-100. The original first dimension gel has been removed and replaced by a stained duplicate (*a*). Note the single precipitin arc produced by the 100000 mol. wt polypeptide. No other precipitin arc was detected (see drawing, *b*).

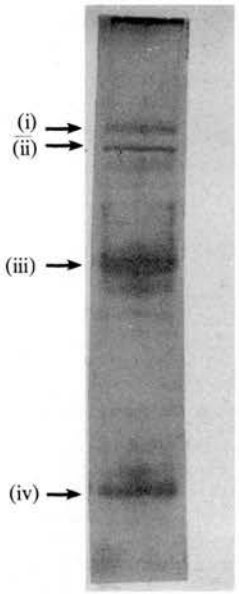

Fig. 6. SDS–PAGE of four pooled excised replicates of the single precipitin line produced by CIE of *B. vulgatus* GNAB 25 OM complex. The arrows indicate (i) 100000 mol. wt major OM polypeptide; (ii) 94000 mol. wt product of IgG; (iii) 55000 mol. wt IgG H-chain; (iv) 23000 mol. wt IgG L-chain.

Visualization of some of the inactivated antigen preparations that reacted with antiserum was performed by CIE. Heat treatment removed all but one precipitin line (line 1). Periodate treatment removed line 1; the other lines remained, but the peak heights of lines 4 and 5 were reduced. Purified LPS gave rise to a double peak that co-precipitated with line 1. There appears to be no cross-reaction between the LPS of *B. vulgatus* NCTC 10583 and the LPS of the other *B. vulgatus* strains examined, i.e. line 1 is produced only by NCTC 10583 antigen. This is in agreement with the observations of Elhag & Tabaqchali (1978) who showed that the O antigens of the *B. fragilis* group (which includes *B. vulgatus*) are extremely heterogeneous within a species. The phenol-extracted capsular polysaccharide produced a small line that appeared to be similar to line 6.

To determine the molecular weight of the species-specific antigen, CIE was performed on *B. vulgatus* GNAB 25 OM complex with SDS–PAGE for the first dimension. GNAB 25 produced only one precipitin line (line 3) in the CIE described earlier in which agarose was the material of the first dimension. By reference to a stained duplicate SDS–PAGE profile, it

was seen that the single line was produced from the major membrane-bound protein of molecular weight about 100000 which was described earlier (Fig. 5). To confirm that the antigen which was demonstrated by CIE was in fact the same antigen that was detected by SDS–PAGE/CIE, the single precipitin line that was produced by GNAB 25 OM complex in CIE was excised from the gel and examined by SDS–PAGE. The immunoprecipitate in agarose from four gels was dissolved in 100 µl SDS–PAGE sample buffer by heating at 100 °C for 5 min and applied molten to a well in an SDS–PAGE gel slab. This produced four major bands, of molecular weights 23000, 55000, 94000 and 100000 (Fig. 6). Bands (iv) and (iii) correspond, respectively, to the L and H chains of IgG. Band (i), of molecular weight 100000, is the major OM protein and band (ii), of molecular weight 94000, is a degradation product of IgG which is produced from purified IgG under the electrophoresis conditions used and is probably a dimer of H chains. It was not derived from the antigen as there was no corresponding band in the GNAB 25 OM complex. From these observations it appears that the species-specific antigen is heat-labile, periodate-stable and is the major outer membrane protein with a molecular weight of about 100000.

Our studies with *B. vulgatus* suggest that it might be possible to define OM protein antigens of other *Bacteroides* species which might be used in serological identification. Such protein antigens would probably be much more constant characters than the capsular antigens that are currently being investigated for *B. fragilis* (e.g. Kasper *et al.*, 1979).

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DETECTION OF SPECIES-SPECIFIC AND CROSS-REACTIVE CELL-SURFACE ANTIGENS OF *BACTEROIDES* SPECIES BY AN INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY

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SUMMARY. EDTA-released outer-membrane antigen complexes were prepared from 172 laboratory and reference strains and 43 fresh clinical or faecal isolates representing 20 species or subspecies of *Bacteroides*, together with 13 species of other genera. These antigens were titrated against antisera to whole, live cells of 21 species or subspecies of Bacteroidaceae in an indirect enzyme-linked immunosorbent assay (ELISA). The presence of species-specific antigens was investigated and cross reactions between species were noted. Results showed that a high proportion of the species possess species-specific antigens with little significant cross reactivity.

We believe that the ELISA system described here detects antigens that represent the whole cell surface of bacteroides organisms. We have exploited the sensitivity of the system and its quantitative potential to define approaches for those wishing to use serological approaches for either the identification of *Bacteroides* species or for the titration of serum antibodies in patients with a possible bacteroides infection.

INTRODUCTION

In the laboratory investigation of infections involving anaerobes at mucosal surfaces, estimation of the numbers of the various organisms present, and a distinction between commensals and potential pathogens, could influence clinical assessment and management. In the development of this expertise, it would be helpful if diagnostic laboratories could identify bacteroides organisms to specific or subspecific level as a routine, but this is still a complex and time-consuming exercise. Although the direct observation of fluorescence of a specimen in ultraviolet light and the direct examination of pus by gas chromatography may be useful adjuncts to direct microscopy and may expedite the detection of anaerobes and the determination of the genera involved, more detailed identification still generally rests upon an extended series of biochemical and other tests (see Duerden *et al.*, 1980).

Serological approaches to detailed identification of *Bacteroides* species and subspecies have been explored by several groups of workers (Beerens *et al.*, 1971;

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Lambe and Jerris, 1976; Abshire, Lombard and Dowell, 1977; Elhag and Tabaqchali, 1978). Commercial kits have been developed (Holland, Stauffer and Altemeier, 1979; Labbé *et al.*, 1980) and an immunoperoxidase method has been described (Hsu *et al.*, 1979). Other workers have explored the possibility of detecting specific antibodies in the serum of patients with bacteroides infections (Hofstad, 1979; Sonnenwirth, 1979).

Our lack of knowledge of the significant surface antigens of these organisms in relation to existing and evolving taxonomic groupings limits the development of serological approaches to the characterisation of gram-negative anaerobes.

Outer membrane (OM) complexes released by EDTA from several species of *Bacteroides* have been shown to contain species-specific antigens that can be demonstrated by enzyme-linked immunosorbent assay (Poxton, 1979). The present study extends this investigation and examines cross reactions that may occur between species.

Materials and methods

The test bacteria included 172 strains representing 20 species or subspecies of *Bacteroides*, five strains of *Capnocytophaga ochracea*, six strains representing four species of *Fusobacterium*, and eight strains representing eight genera of aerobic or facultative gram-negative bacteria.

The following strains were obtained from the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale Avenue, London NW9 5HT: *Bacteroides fragilis* NCTC nos. 9343, 9344, 8560, 10581 and 10584; *B. thetaiotaomicron* NCTC10582; *B. vulgatus* NCTC10583; *B. eggerthi* NCTC11155; *B. splanchnicus* NCTC nos. 10825 and 10826; *B. melaninogenicus* ss. *intermedius* NCTC nos. 9336 and 9338; *B. asaccharolyticus* NCTC9337; *B. corrodens* NCTC10939; *Fusobacterium necrophorum* NCTC nos. 10576 and 10577; *F. necrogenes* NCTC10723; *F. polymorphum* NCTC10562; *F. varium* NCTC10560; *Proteus mirabilis* NCTC6197; *Salmonella abortus equi* NCTC5727; *Shigella sonnei* NCTC8220; *Escherichia coli* NCTC10418; *Pseudomonas aeruginosa* NCTC10662; *Enterobacter aerogenes* NCTC8172; *Branhamella catarrhalis* NCTC3622 and *Haemophilus influenzae* NCTC4560.

B. melaninogenicus ss. *melaninogenicus* ATCC15930 was obtained from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md 20852. *B. distasonis* ATCC8503, *B. ovatus* ATCC8483 and *B. uniformis* ATCC8492 were from Dr Ella M. Barnes, Agricultural Research Council Food Research Institute, Colney Lane, Norwich. *B. variabilis* VPI11368, *B. bivius* VPI6318 and VPI6822, *B. disiens* VPI8057, *B. melaninogenicus* ss. *melaninogenicus* VPI4196 and *B. melaninogenicus* ss. *levi* VPI3300 were obtained from the Virginia Polytechnic Institute and State University (VPI), Blacksburg, Va. 24060. *B. oralis* strain 1210 was obtained from Dr S. Socransky, Forsythe Dental Center, Boston, Mass, and *B. ruminicola* NP333 was obtained from Dr G. H. Bowden, London Hospital Medical College. *B. ureolyticus* WPH58 was obtained from Dr A. L. James, Newcastle Polytechnic, Newcastle upon Tyne. *Capnocytophaga ochracea* (*Ristella ochraceus*) strain 1956c was obtained from Dr M. Sebald, Institut Pasteur, 25 Rue du Docteur Roux, Paris.

Other strains were received in connection with collaborative studies initiated by the ICSB, Taxonomic Sub-committee on Gram-negative Anaerobic Rods (see Holbrook, Duerden and Deacon, 1977; Deacon, Duerden and Holbrook, 1978), or were isolated in this laboratory from clinical material; 43 strains of *Bacteroides* spp. were isolated for use in the fresh isolate study (see below). All strains were identified by the methods of Duerden *et al.* (1976, 1980).

Growth of organisms and preparation of OM antigens was modified from the method of Poxton (1979). Cultures in 50 ml of PPY medium were incubated for up to 48 h to yield good growth. The suspended, washed cells were incubated with 10mM EDTA at 45°C for 2·5 h; after removal of cells by centrifugation, the undialysed supernate was used as the OM antigen.

Antisera were prepared as described by Poxton (1979).

Absorption of antisera. Cross-reacting antibodies were removed by incubating 1 ml of serum with bacteria harvested from 50 ml of an 18-h PPY culture and washed with phosphate-buffered saline. The suspension was shaken during the absorption period of 30 min at 37°C on a

blood-cell suspension mixer. Cells were removed by centrifugation at 20 000 *g* for 30 min at 4°C. The absorption procedure was repeated once.

Micro indirect enzyme-linked immunosorbent assay (ELISA) was modified from that of Poxton (1979). Considerable loss of protein can occur during dialysis and, because dialysed and undialysed OM antigens behaved similarly in the ELISA assay, undialysed preparations were used. No difference in homologous titre was generally found when the OM antigens were used at 30 μg protein/ml or 60 μg protein/ml, so the lower concentration was used. However, for comparison with results already published by Poxton (1979) the 60-μg concentration was used in cross-reactivity studies. The results were read on a Titertek Multiscan Spectrophotometer (Organon Teknika, St Neots, Cambs); the highest dilution of serum that gave an E_{405} value > 1·0 was recorded as the titre.

Study of fresh isolates. Primary isolation plates from clinical specimens that contained anaerobic gram-negative rods, were kindly supplied from the Bacteriology Laboratory, Royal Infirmary of Edinburgh, and the Central Microbiology Laboratories, Western General Hospital, Edinburgh. When possible, a single colony was subcultured into 50 ml of PPY medium and incubated for 18–48 h. This culture was checked for purity, then 5 ml were lyophilised and the remaining 45 ml were used for the preparation of OM antigen. If it was not possible to pick a single colony pure from the primary isolation plate, a subculture was made on BM-based lysed-blood agar containing kanamycin 75 μg/ml and vancomycin 2·5 μg/ml (Holbrook, Ogston and Ross, 1978). After incubation for 48 h, a single colony was subcultured to PPY medium and processed as above.

OM antigens prepared from the fresh isolates were tested initially in a screening assay with selected pools of rabbit antisera before they were titrated against individual sera within any pool. A pool of antisera was prepared by mixing different volumes of several sera so that, when the pool was diluted and tested against its homologous OM antigens, they all reacted to the same titre. The pools were: 1, *B. fragilis* group (BFG) comprising 11 sera; 2a, *B. melaninogenicus* group (BMG, pigmented) comprising four sera; 2b, *B. melaninogenicus* group (BMG, non-pigmented) comprising six sera and 3, asaccharolytic group (AG) comprising three sera.

OM antigens that reacted significantly in screening tests with the BFG pool were further screened against two other pools of BFG sera. They were: 1a, four sera representing three indole-negative *Bacteroides* species and 1b, seven sera representing six indole-positive *Bacteroides* species.

Results

Studies with OM antigens and homologous sera

Outer-membrane antigen preparations from 28 strains representing 23 species or subspecies of non-sporing anaerobes and one strain of *Capno. ochracea* were included in the indirect ELISA with antisera raised against the same strains, except for three of the *Fusobacterium* species, in rabbits and referred to here as species reference sera. OM antigen preparations from the eight control aerobic or facultative species were included in the study. The results are summarised in table I and the figure. Titres in the range 3200–51 200 were recorded for strictly homologous interactions, *i.e.*, when an OM preparation was tested against a reference serum raised specifically against the same strain. Minor heterologous cross reactions were observed in some cases and are indicated as fractions of the homologous titres. When OM antigens from a range of strains within the same species were tested against the species reference antiserum, the results indicated the antigenic homogeneity or heterogeneity of the species (table I). Results obtained with reference and laboratory stock strains are analysed separately from those obtained with freshly isolated strains.

Results with reference and laboratory strains. OM antigen preparations from all *B. fragilis*, *B. vulgatus*, *B. eggerthi*, *B. uniformis*, *B. splanchnicus*, *B. melaninogenicus* ss.

TABLE I

Reactions of OM antigens from reference, laboratory and freshly isolated strains with species reference antisera by indirect ELISA

Species reference antiserum raised against (species and strain no.)	Homologous titre (T)	Number of reference and laboratory strains reacting to a titre of					Number of freshly isolated strains reacting to a titre of					Total number of strains tested	Number of strains reacting to a titre of <T/8
		T	T/2	T/4	T/8	<T/8	T	T/2	T/4	T/8	<T/8		
B. fragilis NCTC9344	12800	8	1	1	...	...	3	17	3	...	1	34	1
B. vulgatus NCTC10583	25600	2	6	2	...	...	...	1	...	...	...	11	0
B. distasonis ATCC8503	12800	2	...	...	...	5	...	...	...	...	1 *	8	2
B. distasonis GNAB26	12800	3	...	1	...	3	...	...	...	...	1		
B. thetaiotaomicron NCTC10582	25600	1	...	1	2	3	...	...	...	2	1	10	2
B. thetaiotaomicron GNAB11	3200	2	2	...	2	1	...	1	1	...	1		
B. ovatus ATCC8483	6400	1	...	...	...	1	...	...	1	...	...	3	1
B. eggerthi NCTC11155	3200	1	4	...	...	...	...	...	...	...	...	5	0
B. uniformis ATCC8492	3200	1	1	1	...	...	...	...	...	...	...	3	0
B. variabilis VPI11368	6400	1	...	...	...	1	...	...	...	...	...	2	1
B. splanchnicus NCTC10826	12800	1	...	1	...	...	...	...	...	...	...	2	0
B. mel. ss. *int.* NCTC9338	12800	5	3	3	...	...	...	...	...	...	...	11	0
B. mel. ss. *levii* VPI3300	3200	1	...	...	...	...	...	...	...	...	...	1	0
B. mel. ss. *mel.* ATCC15930	12800	1	...	...	...	10	...	...	...	...	...	11	5
B. mel. ss. *mel.* VPI4196	25600	1	2	1	1	6	...	...	...	...	...		
B. oralis 1210	51200	3	1	1	...	12	...	...	...	...	...	17	12
B. ruminicola NP333	25600	2	...	1	2	9	...	...	...	...	...	14	8
B. ruminicola GA33	6400	1	...	...	...	13	...	...	...	...	...		
B. bivius VPI6822	51200	1	...	2	...	3	...	...	3	2	4	15	4
B. bivius VPI6318	6400	1	2	...	1	2	2	2	2	1	2		
B. disiens VPI8057	25600	1	1	...	...	...	...	...	...	...	1	3	1
B. asaccharolyticus NCTC9337	25600	1	1	10	11	3†	...	...	...	...	...	27	3†
B. corrodens NCTC10939	3200	1	...	...	...	...	...	...	...	...	...	1	0
B. ureolyticus WPH58	25600	2	...	...	2	...	...	...	...	...	...	4	0
Capno. ochracea 1956C	3200	1	...	...	3	1	...	...	...	...	...	5	1
F. necrophorum NCTC10577	12800	1	...	...	...	1	...	...	...	...	...	2	1

* Results combined when two species reference sera were used.
† Two of these strains are now known to be *B. gingivalis*.

intermedius, and *B. disiens* strains reacted strongly with their respective species reference antisera. Two strains of *B. ureolyticus* reacted less strongly, but the reference serum did not cross react with any other species. Two strains labelled *B. asaccharolyticus* and later shown to be *B. gingivalis* did not react with the *B. asaccharolyticus* antiserum.

When it became apparent that some strains did not react with an antiserum prepared against the same species, sera were raised against other strains in an attempt to find a more representative strain or to determine whether more than one serogroup existed within the species. For example, strains of *B. thetaiotaomicron* reacted more uniformly and more strongly with GNAB11 serum than with NCTC10582 serum, but one strain failed to react with either serum. Two strains of *B. distasonis* reacted with ATCC8503 serum, and four other strains reacted with GNAB26 serum, but one strain failed to react with either serum. These findings indicate that there are two or more serogroups in each of these species. For some species or subspecies the small number of available test strains limited our study.

OM antigens from a significant proportion of the strains of *B. melaninogenicus* ss. *melaninogenicus*, *B. oralis* and *B. ruminicola* failed to react with their species reference antisera. Six of 11 strains of *B. melaninogenicus* ss. *melaninogenicus*, 12 of 17 strains of *B. oralis* and eight of 14 strains of *B. ruminicola* failed to react with their species reference antisera.

Results with freshly isolated strains. After the OM antigens prepared from fresh isolates had been screened against pooled sera (see *Methods*) the following results were obtained with appropriate species reference sera (table I): 23 out of 24 strains of *B. fragilis*, one strain of *B. vulgatus*, two of three strains of *B. thetaiotaomicron*, one strain of *B. ovatus* and seven out of nine strains of *B. bivius* reacted to a titre one-eighth or greater of the respective homologous titres. However, one strain each of *B. fragilis*, *B. distasonis*, *B. thetaiotaomicron* and *B. disiens*, and two strains of *B. bivius* failed to react with their species reference sera. Three strains that failed to react with any group of pooled antisera were later identified as *B. capillosus* for which we do not have an antiserum. The OM antigen prepared from *B. bivius* GNAB136 reacted significantly with more than one serum; it reacted to half of the homologous titre with the species reference serum (VPI6318) and to one-eighth of the homologous titres with the *B. disiens* VPI8057 serum and the *B. oralis* 1210 serum.

Cross reactions between species

B. fragilis group (BFG) OM antigens. When OM antigens from 11 strains representing the nine species comprising the BFG were titrated with rabbit antisera raised against washed whole bacteria, homologous titres were significantly greater and usually at least 16-fold greater than any heterologous cross reactions (figure). However, there were four reactions between BFG OM antigens and BFG sera, in which the difference was 16-fold or less; there were significant cross reactions between a *B. ovatus* antigen and the two *B. thetaiotaomicron* antisera, a *B. uniformis* antigen and a *B. fragilis* serum and a *B. variabilis* antigen and a *B. eggerthi* serum. In addition, three BFG OM antigens cross reacted with *B. melaninogenicus*/*B. oralis*/*B. ruminicola* group antisera to one-sixteenth of the homologous titre. Serum raised against *Capno. ochracea* cross reacted with the OM antigen from *B. distasonis* GNAB26.

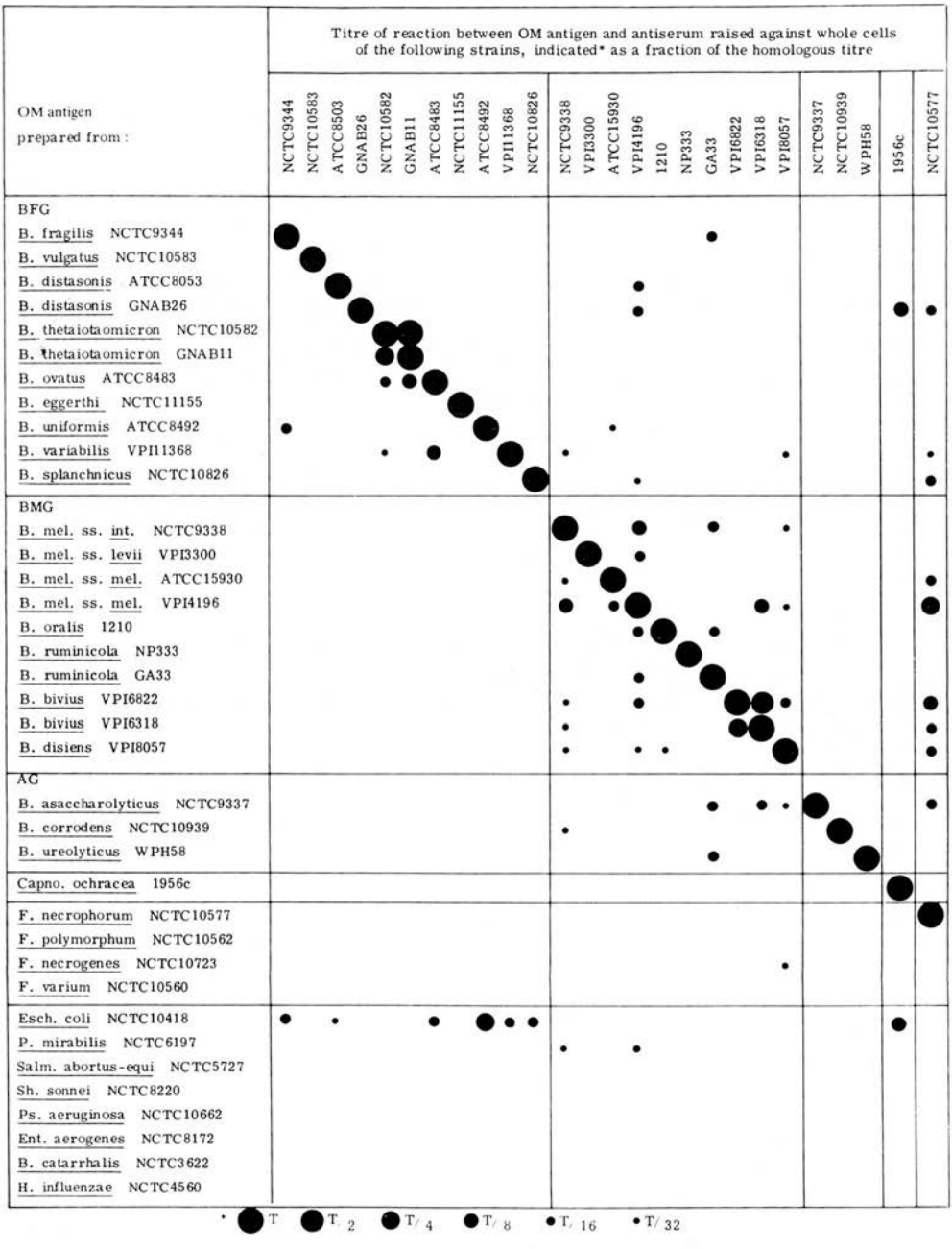

FIG.—Specificity of the interaction of OM antigens and antisera prepared from bacteroides and other organisms in an indirect ELISA system. BFG = *B. fragilis* group; BMG = *B. melaninogenicus/oralis/ruminicola* group; AG = asaccharolytic group.

B. melaninogenicus/oralis/ruminicola group (BMG) OM antigens. The OM antigens of this group did not cross react with antisera raised against species in the BFG or the asaccharolytic group. When these OM antigens were titrated against antisera raised against the BMG species there was usually at least a 16-fold difference between the homologous and cross-reacting titres, but there were 12 cross reactions where the difference was 16-fold or less (figure).

Asaccharolytic group (AG) OM antigens. OM antigens of this group did not cross react with antisera raised against species in the BFG or AG series, but there were some minor cross reactions with antisera raised against the BMG species (figure).

OM antigens of aerobic and facultative gram-negative bacilli. OM antigens from these organisms usually failed to react with the bacteroides antisera or reacted to less than one-sixteenth of the homologous titre. The exception was the OM antigen from *Esch. coli* NCTC10418 which reacted with some of the BFG and AG antisera and the *Capno. ochracea* antiserum to a titre one-sixteenth or greater of the homologous titre, but did not react with the BMG antisera.

Cross reactions with Fusobacterium necrophorum antiserum. Serum raised against *F. necrophorum* cross reacted with many OM antigens prepared from *Bacteroides* spp. but not with the OM antigens of other *Fusobacterium* spp.

Reactions with absorbed antisera. After absorption of cross-reacting antibodies (see *Methods*) the heterologous titres of 10 out of 13 cross-reacting sera were reduced to <400 with no more than a twofold decrease in the homologous titres (table II).

TABLE II

Absorption of cross-reacting antibodies by treatment of antiserum with whole cells of cross-reacting strains

Antiserum raised against strain	Cross-reacting antigen from strain	Titre when homologous antigen was reacted with:		Titre when cross-reacting antigen was reacted with:	
		unabsorbed serum	serum absorbed with whole cells of cross-reacting strain	unabsorbed serum	serum absorbed with whole cells of cross-reacting strain
NCTC9344	ATCC8492	6400	3200	400	<400
NCTC10582	ATCC8483	25600	25600	1600	<400
GNAB11	ATCC8483	3200	3200	400	<400
NCTC11155	VPI11368	6400	3200	400	<400
ATCC8492	NCTC10418	12800	12800	3200	<400
NCTC9338	VPI4196	25600	25600	6400	<400
VPI4196	NCTC9338	51200	51200	3200	<400
VPI4196	ATCC8503	25600	12800	1600	800
VPI4196	GNAB26	25600	12800	1600	800
VPI4196	VPI3300	25600	25600	1600	800
VPI4196	1210	25600	12800	1600	<400
VPI4196	VPI8057	25600	12800	800	<400
1210	VPI8057	25600	12800	1600	<400
VPI6318	VPI4196	12800	6400	1600	<400
VPI8057	NCTC9338	12800	12800	400	<400

DISCUSSION

We have shown here, and previously (Poxton, 1979), that *Bacteroides* species generally have cell-surface antigens that are species specific. The cross reactions that do occur are mainly between similar species; cross-reactive antibodies can be absorbed

with whole cells of the cross-reactive species. Our results suggest that it will be possible to define species-specific surface antigens for many species of *Bacteroides* especially within the *B. fragilis* group. This has already been done for *B. vulgatus* where the species-specific antigen is the major outer-membrane protein (Poxton and Ip, 1981) and for *B. fragilis* where the "capsular" polysaccharide, which appears to be membrane bound and is thus amenable to investigation by ELISA, is the species-specific antigen (Kasper *et al.*, 1977). More strains within the species *B. bivius*, *B. melaninogenicus* ss. *intermedius*, *B. asaccharolyticus* and *B. ureolyticus* also appear to possess species-specific or subspecies-specific antigens. Some species such as *B. melaninogenicus* ss. *melaninogenicus*, *B. oralis* and *B. ruminicola*, are as yet ill defined and there were significant cross reactions within the group; more taxonomic studies must be made before this group can be investigated serologically. For other species, we have not been able to obtain sufficient strains to make a full serological investigation.

In any identification scheme based on serological techniques, the antigens that are to be detected must be clearly defined; their predominance within a species, subspecies or serogroup should be known, and their cross reactions with related and unrelated species must be determined. Moreover, the stability of these antigens and their expression in laboratory-passaged strains must be ascertained. Only one of 24 fresh isolates of *B. fragilis* failed to react with the *B. fragilis* species reference serum. More work is needed to evaluate the significance of the few non-reactors when small numbers of fresh isolates of other species were tested against apparently appropriate species reference sera. A cautious approach to the identification of wild strains by sera raised against stock strains is clearly advisable on principle, but the results with *B. fragilis* strains are particularly encouraging.

The indirect enzyme-linked immunosorbent assay that we used has provided useful results in our investigation of material released from bacteria by mild heat, EDTA and sonication. Our system appears to provide a valid representation of all of the antigens present at the surface of whole cells. The antigens detected could include protein, lipopolysaccharide (LPS) and capsular material; because extremely high dilutions of serum are used, the system will detect the more immunogenic molecules, which have the greatest affinity for the antibodies that we raised against whole cells. Less immunogenic molecules which may be more cross reactive, such as LPS (Elhag and Tabaqchali, 1978), will not be detected at such high dilutions of serum. The extreme sensitivity of the system, its specificity and its quantitative potential will be of use in defining approaches that might exploit immunofluorescence methods for the direct examination of clinical specimens, or might allow the titration of serum antibodies in patients with a possible bacteroides infection.

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Analysis of lipopolysaccharides of *Bacteroides fragilis* by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electroblot transfer

(Silver stain; "Western blot")

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1. SUMMARY

Lipopolysaccharides (LPS) from three strains of *Bacteroides fragilis* were run on SDS-polyacrylamide gels and stained with silver. Each LPS produced a similar pattern, consisting of a series of regularly spaced discrete bands which decreased in intensity as they increased in M_r value. Electroblot transfer from duplicate SDS gels onto nitrocellulose membrane were reacted with antisera raised to whole cells of two of the strains and antigens were visualised with horse-radish peroxidase-anti-rabbit-IgG conjugate and colour reagent. Results revealed that the two lowest M_r bands of the LPS preparation (rough LPS) represented common antigens.

2. INTRODUCTION

B. fragilis, the most commonly isolated anaerobic bacterium from clinical specimens, possessed surface factors, often referred to as "capsule", which appear to confer it with a greater pathogenic potential than that of closely related species [1].

In 1976 Kasper suggested that the capsular polysaccharide may be a species-specific antigen [2] but a recent report has suggested that the earlier preparations were contaminated with LPS and this may have been the species-specific antigen [3]. The presence of several distinct surface carbohydrate antigens has been demonstrated by Hofstad [4] and more recently he has shown by gel filtration that LPS preparations are contaminated with non-LPS carbohydrates [5].

The investigations into the roles of surface components as virulence factors and species-specific antigens have therefore been compromised by attempting to use preparations of unknown purity.

In this study we show that LPS prepared by classical aqueous phenol technique from *B. fragilis* can be analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver. It can also be transferred to nitrocellulose membrane and probed with antisera raised against whole cells of *B. fragilis*. From the results obtained we have been able to comment on the purity of the LPS and suggest the nature of the species-specific antigen.

3. MATERIALS AND METHODS

3.1. Bacterial strains and growth conditions

B. fragilis NCTC9344, and two clinical strains

GNAB4 and GNAB92 which were isolated in our own laboratory, were cultured to late exponential phase in proteose peptone, yeast extract (PPY) medium supplemented with haemin (5 μg. ml^{-1}) menadione (1 μg. ml^{-1}), sodium bicarbonate (0.04%) and cysteine HCl (0.075%) [6] anaerobically with 10% CO_2 at 37°C.

3.2. Preparation of LPS

LPS was extracted from washed, freeze-dried whole bacteria by the aqueous phenol procedure developed by Westphal and Lüderitz [7]. It was purified and washed by two cycles of centrifugation at 100 000 × *g* for 3 h and stored freeze-dried.

3.3. Preparation of antisera

Antisera were raised to whole live washed cells of *B. fragilis* strains GNAB4 and GNAB92 in New Zealand White rabbits by the method of Poxton [8].

3.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

This was performed on 10% slab gels with the buffer system of Laemmli [9] by the method described by Poxton and Brown [10]. Gels were stained with silver after periodic acid treatment by the method of Tsai and Frasch [11] and also with Coomassie Blue [12].

3.5. Electro-blot transfer and enzyme immunoassay

This was essentially by the method of Towbin et al. [13]. Material was transferred to nitrocellulose membrane (Transblot™ Transfer Medium, BioRad) in Tris, glycine, methanol buffer, pH 8.3 [13] over a 16-h period at 12 V, 40 mA.

The transferred antigens were detected with the ImmunBlot™ immunoassay (BioRad). Briefly, the membrane, after washing for 10 min in Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5, TBS) was placed in 3% gelatin TBS for 45 min to block any unbound sites. It was then transferred into the *B. fragilis* antiserum diluted 1 : 100 in 1% gelatin TBS and incubated for 3 h at room temperature. After two-min washes in 0.025% Tween-20 TBS the membrane was placed into goat anti-rabbit IgG-horse radish peroxidase conjugate (BioRad) diluted 1 : 3000 in 1% gelatin TBS and incubated for 1 h at room temperature. After a further two 10-min washes in Tween TBS it was placed into HRP colour development solution (BioRad) which contains 4-chloro-1-naphthol and hydrogen peroxide. Colour development took place between 5 and 15 min. The reaction was stopped by placing into distilled water and washing with several changes of water over 1 h. All of the above steps were performed in clean glassware and with gentle agitation throughout.

3.6. Carbohydrate assay

This was performed on the LPS preparation by the method of Dubois et al. [14] with glucose as the standard.

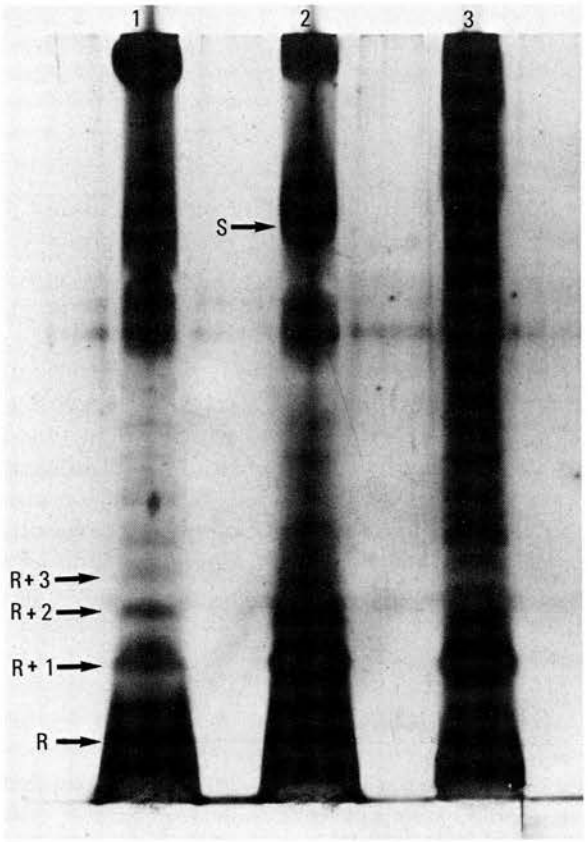

Fig. 1. SDS-polyaclyamide gels of LPS (20 μg) from *B. fragilis* NCTC9344 (track 1), GNAB92 (track 2) and GNAB4 (track 3) stained with silver after periodic acid oxidation. S represents possible smooth LPS and R is rough LPS. R+1, 2, etc. represents rough LPS with increasing numbers of repeating units.

4. RESULTS

The three LPS preparations were run on SDS-PAGE: the gel was loaded with 20 μg (based on carbohydrate assay) of each LPS and stained with silver; the final development proceeding for no more than 5 min. A significantly shorter development resulted in clearer definition of the heavier staining bands but the finer bands were not visible. Conversely a longer development time increased the sensitivity but results were difficult to read. The results are shown in Fig. 1. Each LPS preparation gave a similar pattern. At the front of each track there was an almost clear area where stain had not penetrated and just behind this there was a series of regularly spaced discrete low-M_r bands which decreased in staining intensity as they approached the middle of the gel. This is best seen in track 1 (NCTC9344 LPS). At the top half of each track there were three high-M_r diffuse-stain-

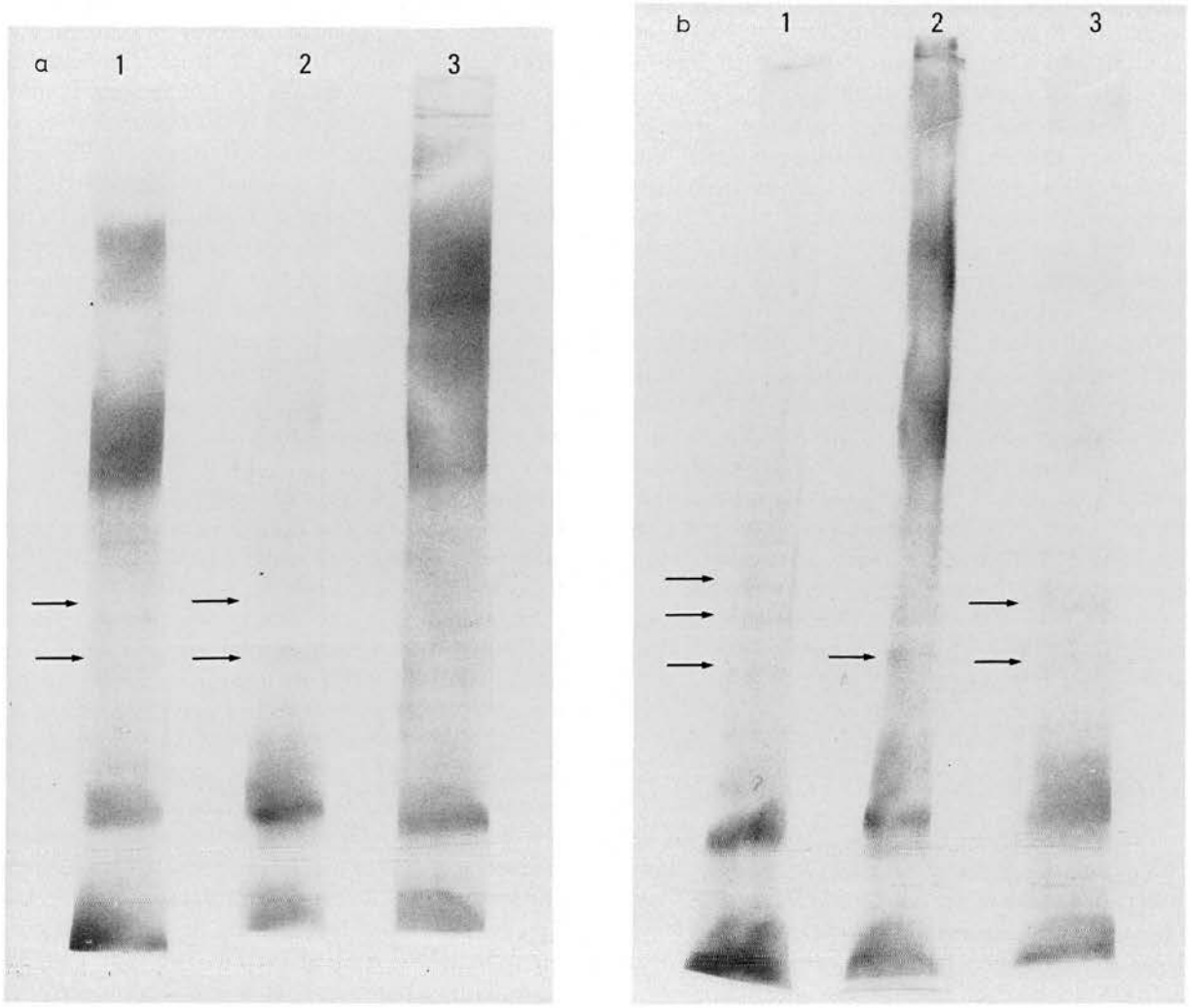

Fig. 2. Electroblots of LPS (20 μg) from NCTC9344 (track 1), GNAB92 (track 2) and GNAB4 (track 3) which had been separated on SDS gels as in Fig. 1 and transferred to nitrocellulose membranes. Reaction with antiserum raised against (a) GNAB4 and (b) GNAB92, followed by anti-rabbit IgG and HRP colour development (BioRad). Faint reactions are arrowed.

ing bands. On closer examination the middle diffuse band in track 2 (GNAB 92 LPS) can be seen to be composed of a series of closely spaced discrete bands (arrowed → S).

To estimate the protein in the LPS preparations the amount of sample on the gels was increased from 20 μg to 50 μg (carbohydrate) and stained with Coomassie Blue. (We have found the Lowry protein estimation to be of little use on LPS preparations from bacteroides due to the interference from amino sugars and other unidentified components. Apparent high protein levels give no Coomassie Blue bands on gels). After staining with Coomassie Blue a grey-blue region was visible at the front which corresponded to the clear area in the silver stain and just behind this there was a faintly stained band which may have been protein. Other than this no definite bands were visualised.

Electroblot transfer was performed from two duplicate gels which contained 20 μg of each LPS. One blot was probed with *B. fragilis* GNAB4 antiserum (Fig. 2a) and the other with *B. fragilis* GNAB92 antiserum (Fig. 2b). In Fig. 2a the GNAB4 antiserum reacted strongly with both high and low-M_r bands in the homologous reaction (track 3) and also in the reaction with NCTC9344 LPS (track 1). However, with GNAB92 LPS only the two lowest M_r bands reacted strongly, but several faint bands of increasing M_r were visible (track 2). In Fig. 2b the homologous reaction (track 2) demonstrated a reaction between both high and low-M_r components, but with the two heterologous reactions only the two lowest M_r weight bands reacted strongly but, as in Fig. 2a, faint higher-M_r bands were just discernible.

5. DISCUSSION

The silver-stained SDS-polyacrylamide gels of LPS from the three strains of *B. fragilis* show similarities to those previously reported for *E. coli, Salmonella typhimurium, Neisseria meningitidis* and *Bordetella pertussis* [11.15]. They are also similar to the SDS gel profiles revealed by autoradiography of LPS from *E. coli* and *S. typhimurium* [16,17]. The presence of multiple, regularly spaced bands has been interpreted as LPS molecules possessing increasing numbers of O-antigen-repeating units. The heavily stained low-M_r component represents rough LPS (i.e. lipid A and core) and each band of increasing M_r represents the addition of one more repeating unit (these have been indicated on Fig. 1, track 1 as R, R + 1, R + 2, R + 3, etc.). This interpretation probably also applies to the LPS of *B. fragilis*. The material at the absolute front of the gel, which did not stain well, may represent the lipid A component which does not stain with silver [11].

The diffuse-staining high-M_r bands have not been so apparent in PAGE analyses of the enterobacterial LPS preparations. The middle of the three high-M_r bands in Fig. 1, track 2, labelled S may be a series of smooth LPS molecules. It may be possible that the other bands represent contaminating non-LPS carbohydrates; either capsular polysaccharide or the high-M_r carbohydrates described by Hofstad [5]. In preliminary work in our laboratory, capsule preparations have been shown to have apparent high M_rs on SDS gels.

Electroblot transfer of the three LPS preparations and subsequent detection of antigens by antisera raised to whole cells of the two clinical isolated revealed that the only components which reacted significantly with both antisera were the two lowest-M_r bands (R and R + 1) although faint reactions were visible with some of the higher-M_r bands (arrowed). The homologous reactions also detected the high-M_r components. Some of the high-M_r bands of the reference strain (NCTC9344) also reacted with the GNAB4 antiserum. These observations demonstrate that the probable core region of the LPS is an antigen common to the three strains of *B. fragilis* and may represent a *B. fragilis* common antigen. If we assume that the middle high-M_r band (S in Fig. 1) represents smooth LPS then a likely explanation for only the rough and semirough LPS reacting with antiserum is either because of a concentration effect i.e. there are only sufficient core determinants present in the first two bands to be detected, or the presence of many repeating units mask the core. The high-M_r carbohydrates are not species-specific antigens but may be shared by more than one strain. This is in agreement with the work of Kasper et al. [3] who have found LPS from two strains of *B. fragilis* to

have an extremely similar chemical composition and to be identical antigenically by use of inhibition of an enzyme-linked immunosorbent assay.

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Crossed Immunoelectrophoresis and Enzyme-linked Immunosorbent Assay of the Cell-surface Antigens of *Bacteroides fragilis*

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Antisera were raised to whole, live cells of a reference strain (NCTC 9344) and two clinical isolates (GNAB 92 and GNAB 4) of *Bacteroides fragilis*. Each antiserum was reacted in crossed line immunoelectrophoresis (CLIE) with EDTA-heat-sonication-prepared outer membrane (OM) complex from 10 *B. fragilis* strains. In addition, the antisera were reacted with these antigens in an enzyme-linked immunosorbent assay (ELISA). In CLIE, the antisera raised to the reference strain and one of the clinical isolates (GNAB 92) demonstrated a heat labile antigen which was common to all 10 of the test strains. Lipopolysaccharide (LPS) prepared from both the clinical isolates produced three major precipitin lines when reacted with their homologous antisera in crossed immunoelectrophoresis (CIE). In both cases, these three antigens were present as major components of the OM complex. Each antiserum reacted significantly in ELISA with all test OM complex preparations. Inhibition of ELISA showed that carbohydrates were the predominant cross-reactive antigens in ELISA and that in the case of the clinical isolate GNAB 4, most of the cross-reactive antigenic activity was present in the homologous LPS preparation.

INTRODUCTION

Bacteroides fragilis is the most common anaerobic organism isolated from clinical specimens (Wren *et al.*, 1977). It is a significant pathogen in various wound infections, especially those of the abdomen (Finegold, 1977). It can be found either in pure culture or more frequently together with facultative organisms. *Bacteroides fragilis* has surface components, usually referred to as capsule, which apparently make it more virulent than the more common faecal commensal bacteroides, e.g. *B. vulgatus*, to which it is closely related (Onderdonk *et al.*, 1977). These observations, and the subsequent need for a rapid and more reliable sero-identification scheme, have led to several studies of the cell surface of the species.

Methods used to examine the surface antigens of *B. fragilis* have most often concentrated on heat stable carbohydrate components and have included tube agglutination of whole, boiled cells (Lambe & Moroz, 1976; Elhag & Tabaqchali, 1978) and immunodiffusion of cell extracts (Abshire *et al.*, 1979; Cherniak *et al.*, 1979). The antigenic properties of purified lipopolysaccharide (LPS) have also been studied (Hofstad, 1975, 1977; Meizel-Mikolajczyk *et al.*, 1981). All investigations have shown complex patterns of cross-reactions and thus considerable antigenic diversity associated with heat stable antigens.

The role of individual cell surface components as antigens is uncertain. Kasper & Seiler (1975) and Kasper (1976) isolated a high molecular weight, species-specific capsular polysaccharide antigen, but Kasper *et al.* (1983) have reported that the purified substance was a mixture of LPS and capsule. Recent studies have also indicated that LPS purified from *B. fragilis* by the aqueous phenol method of Westphal & Luderitz (1954) may be contaminated with non-O-antigenic, carbohydrate precipitating antigens (Hofstad, 1981, 1982).

Abbreviations: CIE, crossed immunoelectrophoresis; CLIE, crossed line immunoelectrophoresis; ELISA, enzyme-linked immunosorbent assay; OM, outer membrane.

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An enzyme-linked immunosorbent assay (ELISA) has been developed which detects species-specific antigens in *B. fragilis* outer membrane preparations (Poxton, 1979; Poxton *et al.*, 1982). However, the relationship of the ELISA antigens to LPS, other carbohydrate antigens and outer membrane proteins is not known. In this study, the nature of the antigens detected by ELISA was investigated by the inhibition of ELISA technique. Crossed immunoelectrophoresis (CIE) was also performed to investigate the relationship between precipitating antigens and the antigens detected by ELISA.

METHODS

Culture of bacteria and preparation of EDTA-outer membrane complex. Bacteroides fragilis NCTC 9344 and nine laboratory isolates (GNAB 4, 85, 86, 87, 88, 90, 92, 97 and 98) that had been previously characterized according to the criteria of Duerden *et al.* (1980) were cultured anaerobically from freeze-dried inocula in PPY medium (Holbrook *et al.*, 1977). Outer membrane (OM) complexes were prepared by the EDTA-heat-sonication treatment previously described (Poxton & Brown, 1979), except that incubation with EDTA buffer was extended from 30 min to 2 h and all samples were concentrated in an ultrafiltration cell with a PM-10 membrane (Amicon Corp., Lexington, Mass., USA).

Lipopolysaccharide (LPS) preparation. LPS was extracted from freeze-dried bacteria that had been grown in PPY medium for 18 h by the aqueous phenol procedure developed by Westphal & Luderitz (1954). It was purified and washed by centrifugation at 100000 *g* for 3 h.

Treatment of OM complex. Separate samples of EDTA-released OM complex at a concentration of 0·5–1·0 mg protein ml^{-1} were treated with: (*a*) Heat (121 °C) for 15 min.; (*b*) 0·1 M-sodium periodate in sodium acetate buffer (pH 5) at 20 °C overnight. Excess periodate was consumed by the addition of ethylene glycol and samples were dialysed against 0·15 M-phosphate buffer pH 7·4 (PBS). A control sample was taken through each stage with the omission of sodium periodate. (*c*) A mixture of pronase (BDH) and trypsin (Sigma) both at a concentration of 0·1 mg ml^{-1}, overnight at 37 °C. A control sample was also incubated at 37 °C without the enzymes.

Preparation of antisera. Antisera were raised against washed live cells of *B. fragilis* NCTC 9344, *B. fragilis* GNAB 4 and *B. fragilis* GNAB 92 in New Zealand White rabbits as previously described (Poxton, 1979).

Enzyme-linked immunosorbent assay (ELISA). Indirect ELISA was carried out as previously described (Poxton & Byrne, 1981). Briefly, OM complex was diluted to 30 μg protein ml^{-1} in pH 9·6 carbonate buffer and coated onto wells of microtitre plates. After reaction with rabbit antiserum, anti-rabbit-IgG–alkaline phosphatase conjugate (Miles Laboratories, Stoke Poges, Slough, UK) and substrate (*p*-nitrophenyl phosphate, Sigma) results were read in a Titertek Multiskan (Organon Teknika, St Neots, Cambs., UK). The end-point of the titration (titre) was the first dilution of serum with an A_{405} value of less than 1·6. An ELISA inhibition test was performed by pre-incubating antisera with potential inhibitors: antiserum at four or eight times its titre, depending on strength of heterologous reaction, was incubated with an equal volume of doubling dilutions of untreated, heat treated or periodate treated OM complex (2 mg protein ml^{-1}) or LPS (400 μg carbohydrate ml^{-1}). A 1 in 2 dilution of OM complex or LPS was the first inhibitor concentration used. Absorbance (A_{405}) readings for each inhibited reaction were expressed as a percentage of the normal uninhibited reaction.

Crossed immunoelectrophoresis (CIE) with intermediate gel. The procedure was essentially that described for CIE by Weeke (1973). OM complexes were adjusted to a concentration of 2 or 3 mg protein ml^{-1} in PBS containing 1% (v/v) Triton X-100. These antigens (16 μl) were examined against homologous antisera in CIE on sheets (5 × 5 cm) of GelBond (Marine Colloids, Rockland, Maine, USA). Gel and electrophoresis buffer composition were as previously described (Poxton & Byrne, 1981). Electrophoresis was at 12·5 V cm^{-1} for 1·5 h in the first dimension and at 12 V cm^{-1} for 16 h in the second dimension, both at 4 °C. Gels were washed, pressed and stained with Coomassie blue. A 5 × 1 cm agarose strip (intermediate gel) containing antiserum (or nothing in controls) was incorporated between the first and second dimension gels.

Crossed line immunoelectrophoresis (CLIE). The conditions for CLIE were as described above for CIE except that the intermediate gel contained heterologous OM antigen preparations (150–450 μg protein). This technique detects cross-reactions between the homologous antigens run in the first dimension and heterologous antigens contained in the intermediate gel (Krøll, 1973).

Analytical techniques. Protein concentrations were estimated by the Lowry method, and carbohydrate as glucose equivalents was estimated by the method of Dubois *et al.* (1956).

RESULTS

CIE and CLIE

Crossed immunoelectrophoresis of OM complex from *B. fragilis* strains NCTC 9344 (reference strain), GNAB 92 and GNAB 4 (clinical isolates) was performed against homologous antisera.

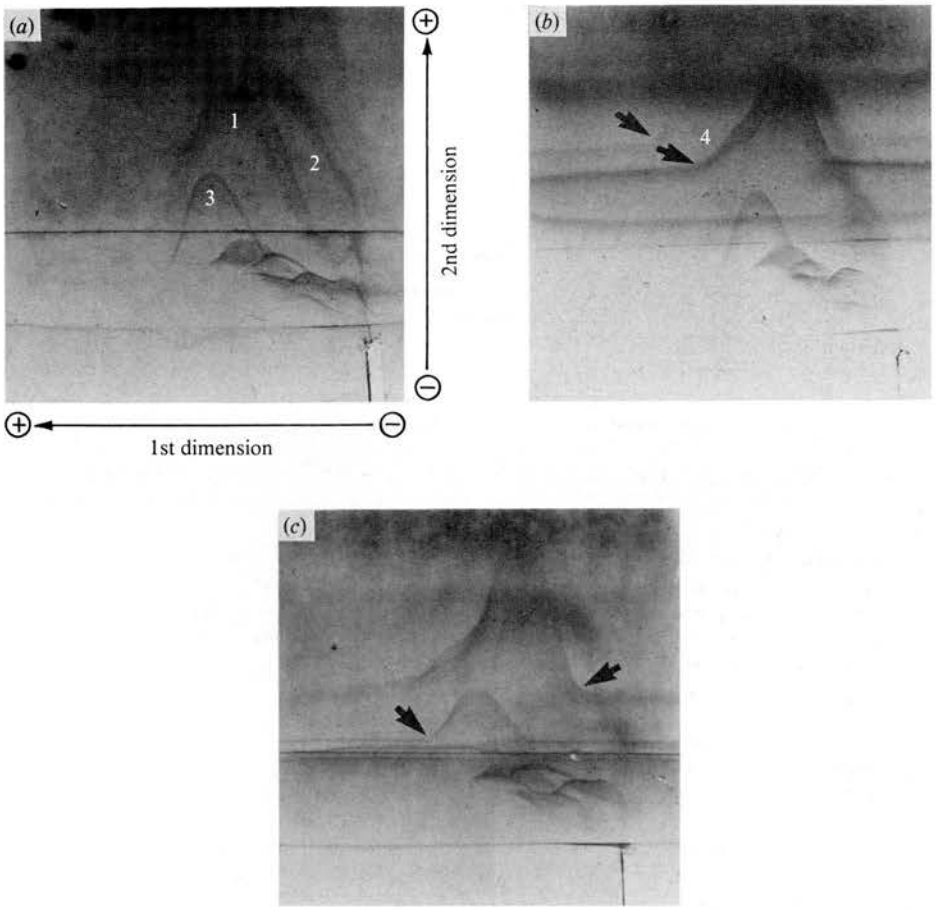

Fig. 1. CIE and CLIE reactions of 9344 antiserum: (*a*) CIE of 9344 OM complex with blank intermediate gel; (*b*) CLIE of 9344 OM complex with GNAB 85 OM complex in the intermediate gel; (*c*) as in (*b*) except with GNAB 90 OM complex in the intermediate gel. In the first dimension 16 μl NCTC 9344 OM complex (48 μg protein) was run, against 500 μl antiserum in the second dimension. Intermediate gels (*b* and *c*) contained 450 μg protein in 0·75 ml agarose gel. The numbers indicate the precipitin arcs of cross-reacting antigens.

The presence of cross-reacting precipitating antigens in heterologous strains was detected by inclusion of heterologous OM complex in an intermediate gel (CLIE).

Reactions with NCTC 9344 antiserum. The homologous reaction with NCTC 9344 OM complex produced several precipitin arcs (Fig. 1 *a*), and those that were subsequently proved to be cross-reacting antigens are numbered. CLIE was done with OM antigens from the nine heterologous test strains. The results obtained with OM complex from GNAB 85 and GNAB 90 in intermediate gels are shown in Fig. 1 (*b*, *c*). Appearance of a horizontal precipitin line indicated a reaction between the NCTC 9344 antiserum and a heterologous antigen, and the joining of such a line with a homologous precipitin arc indicated a cross-reaction between the antigens. Cross-reaction of the GNAB 85 OM complex with antigen 1 can be seen in Fig. 1 (*b*) (arrowed). This strain also cross-reacted with a faint precipitate that was not visible in the control reaction but which became apparent in CLIE reactions (arrowed). This antigen was numbered 4 (Fig. 1 *b*). In Fig. 1 (*c*) it can be seen that the OM preparation from GNAB 90 cross-reacted with antigens 1 and 3 (arrowed). The nine test strains varied in their cross-reactions with antigens 2, 3 and 4, but all strains possessed an antigen that cross-reacted with antigen 1,

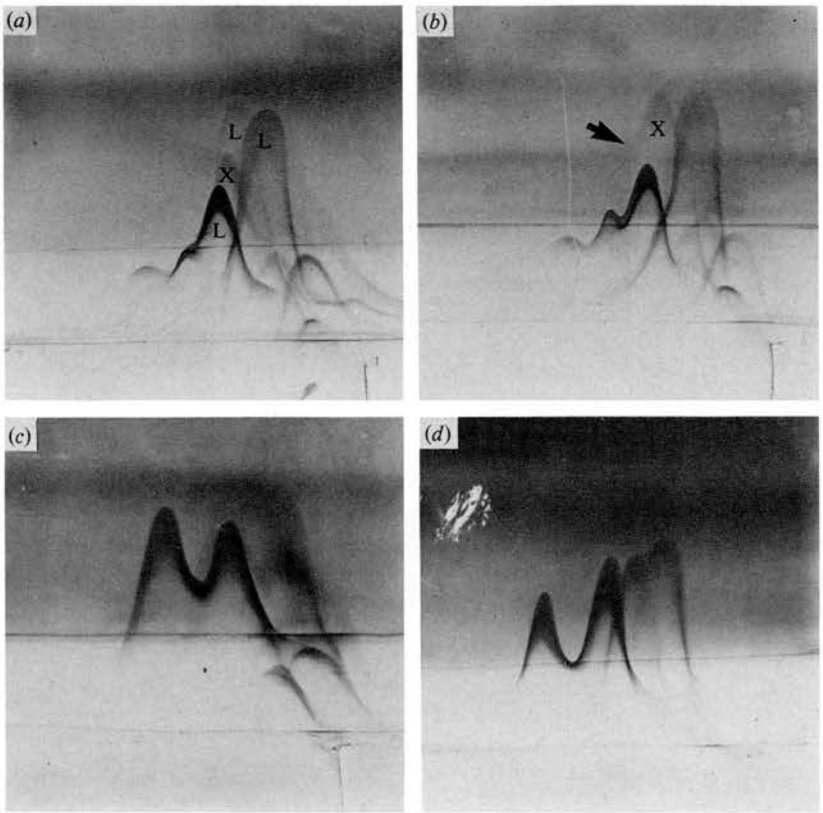

Fig. 2. CIE and CLIE reactions of GNAB 92 antiserum: (*a*) CIE of GNAB 92 OM complex with blank intermediate gel; (*b*) CLIE of GNAB 92 OM complex with GNAB 87 OM complex in the intermediate gel; (c) CIE of heated GNAB 92 OM complex with blank intermediate gel; (*d*) CIE of GNAB 92 LPS with blank intermediate gel. In the first dimension 16 μl GNAB 92 OM complex (48 μg protein) or LPS (4 μg carbohydrate) was run, against 375 μl antiserum in the second dimension. Intermediate gel (*b*) contained 150 μg protein in 0·75 ml agarose gel. L and X mark the cross-reacting antigens.

indicating the presence of a *B. fragilis* common antigen. Periodate treatment of the NCTC 9344 OM preparation had little effect on the homologous picture and the antigen 1 precipitate was unmodified. Heating the OM complex (121 °C for 15 min) destroyed most of the antigens although some ill-defined precipitates remained. It was unclear which of the antigens these faint reactions represented. The damage to antigen 1 by heat and its resistance to periodate suggested that it may be a protein. When the OM complex was treated with pronase and trypsin, the antigen 1 precipitate became faint and cross-reacted poorly in CLIE indicating that antigen 1 is a protein.

Purified NCTC 9344 LPS did not react strongly with the antiserum, but did produce faint precipitates representing at least two distinct antigens.

Reactions with GNAB 92 antiserum. The homologous reaction with GNAB 92 OM complex is shown in Fig. 2(*a*). Outer membrane complexes from the nine heterologous test strains were included in intermediate gels and an example of the CLIE pattern obtained with GNAB 87 is shown in Figure 2(*b*). A cross-reaction with the antigen marked X in Figure 2(*a*) occurred (arrowed). The antigen X precipitate joined up with the horizontal precipitin line and was positioned higher in the gel than in the control. The OM antigens from the other eight heterologous strains also cross-reacted with antigen X. Most of the strains only showed cross-reaction with this single antigen. Periodate treatment of the GNAB 92 OM complex destroyed all the major antigens, including antigen X. Several antigens were resistant to heat (Fig. 2*c*),

although antigen X was destroyed. This is difficult to see from Fig. 2(*c*), but the destruction of antigen X was confirmed by running a CLIE with untreated homologous antigen in the first dimension and heated heterologous OM complex in the intermediate gel (i.e. as Fig. 2*c* but unheated): no line precipitate occurred. Treatment with pronase and trypsin hardly affected the picture, although the mobility of antigen X towards the anode increased slightly.

The results indicated that there are several distinct carbohydrate antigens associated with the GNAB 92 OM complex. Also, a heat labile, periodate sensitive common antigen (X), possibly a glycoprotein, exists. Purified GNAB 92 LPS produced three distinct antigens in CIE (Fig. 2*d*). When LPS was incorporated into the intermediate gel of the homologous OM complex reaction, it cross-reacted with the three antigens marked 'L' in Figure 2(*a*).

Reactions with GNAB 4 antiserum. The homologous reaction with GNAB 4 OM complex yielded several precipitates (Fig. 3*a*). In contrast to the results obtained with the NCTC 9344 and GNAB 92 antisera, CLIEs with GNAB 4 antiserum did not demonstrate a common antigen in the ten test strains. The OM complex from strain GNAB 86 cross-reacted with antigen A (Fig. 3*b*, arrowed) as did strain GNAB 97. Strains NCTC (Fig. 3*c*, arrowed) and GNAB 87 cross-reacted with antigen C and appear to have cross-reacted weakly with antigen A. Strain GNAB 98 was the only strain to cross-react with antigen B. Cross-reactions of OM complexes from strains GNAB 85, GNAB 88, GNAB 90 and GNAB 92 could not be demonstrated by CLIE, even when levels of 600 μg protein were included in the intermediate gel. Periodate treatment destroyed most of the antigenic activity of the GNAB 4 OM complex and at least three of the antigens were resistant to heat (Fig. 3*d*).

Purified GNAB 4 LPS was run against homologous antiserum (Fig. 3*e*): two heat resistant antigens were detected. The GNAB 4 OM complex was run against homologous antiserum with LPS in the intermediate gel. The CLIE pattern obtained confirmed the presence of antigens A and C in LPS but also detected two other antigens. One of these cross-reacted with antigen B but the other showed no cross-reaction with the OM complex precipitates.

The results indicated that GNAB 4 antiserum reacted with several carbohydrate antigens in the homologous OM complex. Four precipitating antigens were detected in the LPS preparation, three of which were the major cross-reacting antigens. The presence of four precipitating antigens in ultracentrifuged LPS indicates co-purification of antigens other than LPS.

The antisera to NCTC 9344 and GNAB 92 had demonstrated a common antigen in the ten test strains (antigen 1 and antigen X). They were both heat labile but their sensitivity to periodate differed. In an attempt to determine if they were in fact the same antigen, CIEs with antiserum-containing intermediate gels were run. Antigen from GNAB 92 was run through an intermediate gel containing GNAB 9344 antiserum into GNAB 92 antiserum (Fig. 4). When compared to the control CIE without antiserum in the intermediate gel (Fig. 2*a*) precipitin line X is seen to have precipitated in the intermediate gel. In the converse experiment, NCTC 9344 antigen run through GNAB 92 antiserum, a similar effect was seen with line 1 (result not shown due to difficulty in reproduction). This demonstrated that there were antibodies to the common antigens in both heterologous antisera, suggesting circumstantially that these common antigens shared determinants.

Enzyme-linked immunosorbent assay (ELISA)

The three test antisera were reacted in ELISA with OM complex from each of the ten test strains (Table 1). In only three instances did a heterologous reaction fall below an eighth of the homologous titre, thus indicating strong ELISA reactions of the three antisera with heterologous strains.

Inhibition of ELISA. Inhibition studies were performed to investigate the nature of the antigens that reacted in ELISA. Potential inhibitors of the ELISA reactions were (*a*) homologous untreated (i.e. positive inhibition control), (*b*) heat treated and (*c*) periodate treated OM complexes. Antisera were incubated with doubling dilutions of potential inhibitors prior to

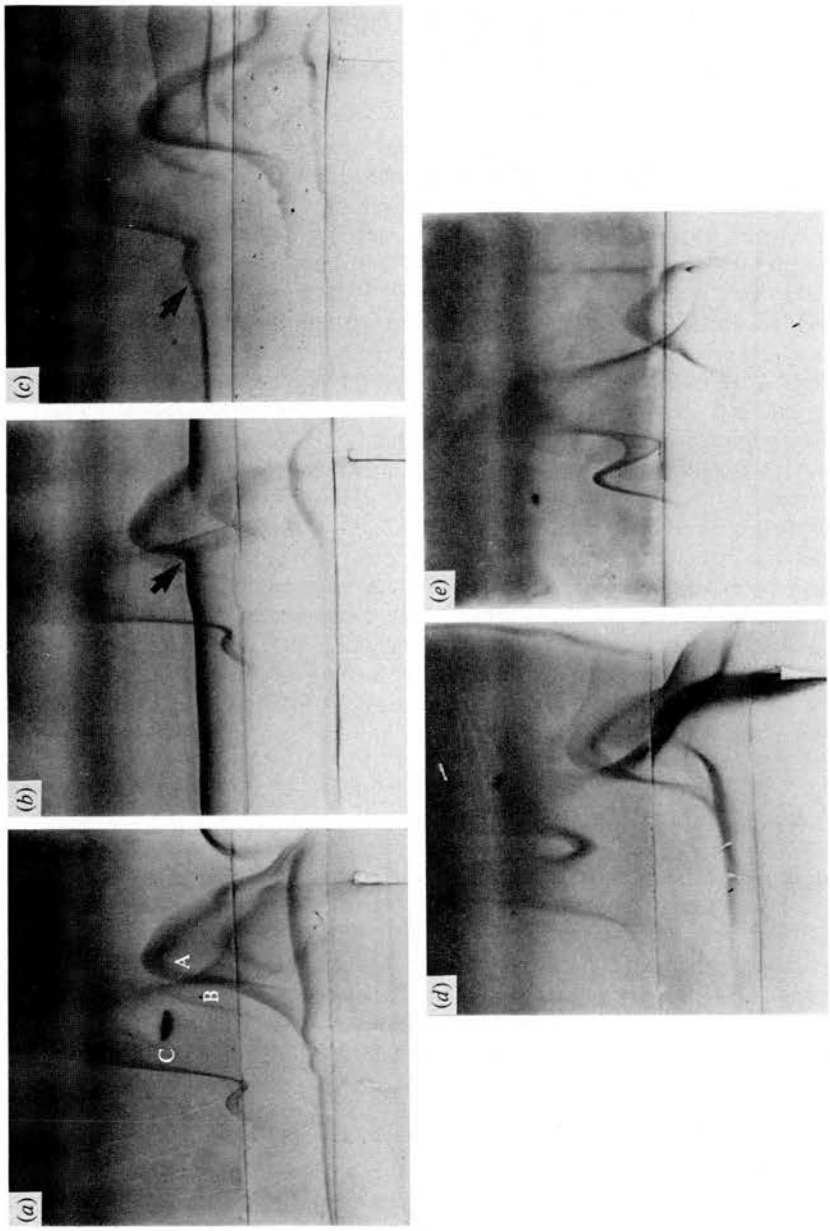

Fig. 3. CIE and CLIE reactions of GNAB 4 antiserum: (*a*) CIE of GNAB 4 OM complex with blank intermediate gel; (*b*) CLIE of GNAB 4 OM complex with GNAB 86 OM complex in the intermediate gel; (*c*) as in (*b*) except with 9344 OM complex in the intermediate gel; (*d*) CIE of heated GNAB 4 OM complex with blank intermediate gel; (*e*) CIE of GNAB 4 LPS with blank intermediate gel. In the first dimension 16 µl GNAB 4 OM complex (32 µg protein) or LPS (6 µg carbohydrate) was run, against 375 µl antiserum in the second dimension. Intermediate gels (*b* and *c*) contained 150 µg protein in 0·75 ml of agarose gel. A, B and C mark the cross-reacting antigens.

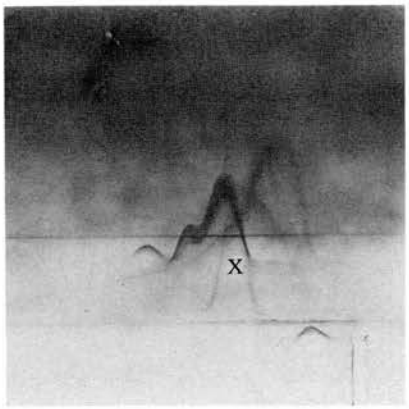

Fig. 4. CIE of GNAB 92 OM complex with NCTC 9344 antiserum in intermediate gel. 16 µl (48 µg protein) of GNAB 92 OM complex was run in the first dimension, through an intermediate gel containing 125 µl NCTC 9344 antiserum (in 0·75 ml agarose), into 375 µl of GNAB 92 antiserum in the second dimension. X marks the precipitin line which precipitated in the intermediate gel.

Table 1. *Reactions of Bacteroides fragilis OM antigens with the three test antisera*

The titre is defined as the first dilution of serum with an A_{405} value of less than 1·6. The titre of the homologous reactions is marked (T).

Antigen prepared from strain no.:	Titre of given antigen when titrated against antiserum raised against:		
	NCTC 9344	GNAB 4	GNAB 92
NCTC 9344	12800 (T)	6400	3200
GNAB 4	3200	25600 (T)	6400
GNAB 85	3200	6400	6400
GNAB 86	6400	6400	6400
GNAB 87	3200	6400	6400
GNAB 88	1600	1600	800
GNAB 90	12800	3200	6400
GNAB 92	6400	6400	25600 (T)
GNAB 97	6400	3200	3200
GNAB 98	12800	1600	6400

addition to the ELISA plate wells. In all experiments, each of the uninhibited reactions (as in Table 1) was included as a control. Absorbance readings in the inhibited reactions were expressed as a percentage of the normal uninhibited ELISA reaction.

Inhibition of NCTC 9344 antiserum reactions. The extent to which heat or periodate treatment reduced the capacity of NCTC 9344 OM complex to inhibit the ELISA reactions of NCTC 9344 antiserum varied, depending on the test antigen. However, three trends occurred in the results and examples of each trend are shown in Fig. 5(*a–c*). Inhibition of the reaction with GNAB 87 OM complex produced results (Fig. 5*a*) representative of those obtained with five other strains (GNAB 4, 85, 87, 90 and 98). The weak inhibition by periodate treated antigens showed that carbohydrates were the major antigens in these reactions. The position of the curve showing inhibition by heated OM complex indicated that both heat resistant and heat labile antigens took part in the reactions. Inhibition of the homologous (NCTC 9344) and GNAB 92 reactions exhibited a second trend (Fig. 5*b*): inhibition by heated antigens was similar to that found in the first trend, but periodate treated antigens inhibited to a greater extent than in the first trend. The third trend occurred in the reactions with strains GNAB 88 and GNAB 97 (Fig. 5*c*) and showed

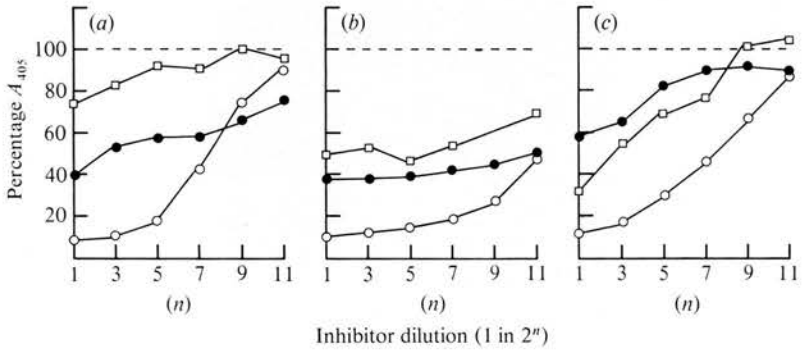

Fig. 5. Inhibition of ELISA reactions of NCTC 9344 antiserum by untreated (○), heated (●) and periodate treated (□) homologous OM complex. (*a*), reactions with GNAB 87 OM complex; (*b*), reaction with GNAB 92 OM complex; (c), reaction with GNAB 97 OM complex. 100% absorbance was that obtained in the control reaction, i.e. no inhibitor added to antiserum.

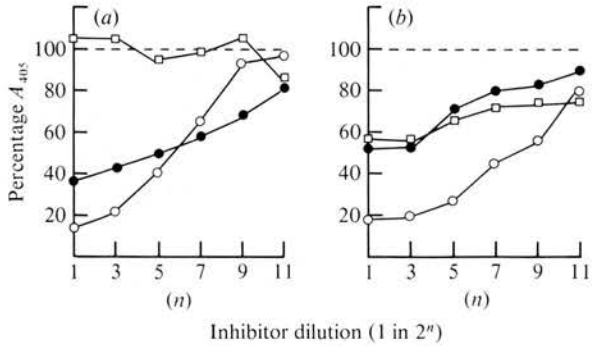

Fig. 6. Inhibition of ELISA reactions of GNAB 92 antiserum by untreated (○), heated (●) and periodate treated (□) homologous OM complex. (*a*), reactions with GNAB 98 OM complex; (*b*), reactions with GNAB 88 OM complex.

that heat labile antigens were of greater importance than in the reactions with other strains. The results suggested that reactions showing the second and third trends may have involved important protein antigens as well as carbohydrates.

Inhibition of GNAB 92 antiserum reactions. Two general trends were found in the reactions with GNAB 92 antiserum. The results of the reactions with OM complex from strain GNAB 98 (Fig. 6*a*) are an example of the pattern obtained with seven other strains, including the homologous strain. In the GNAB 98 reaction, negligible inhibition occurred with periodate treated inhibitor. In four of the reactions showing this trend, some inhibition occurred at the two lowest inhibitor dilutions, but this became negligible on further dilution. The position of the curve showing inhibition by heated antigens indicated that heat resistant antigens were very important in ELISA, while heat labile antigens were involved to a lesser extent. It is unclear why the heated antigens inhibited ELISA less than untreated antigens at low dilutions and more at high dilutions, but this was a consistent finding in the eight reactions. These results indicated that in eight out of the ten ELISA reactions of GNAB 92 antiserum, carbohydrates were the major antigens.

A second trend occurred in the reactions with strains GNAB 88 (Fig. 6*b*) and GNAB 97. Heat labile, periodate resistant antigens had a significant role in ELISA, suggesting importance of protein as well as carbohydrate antigens.

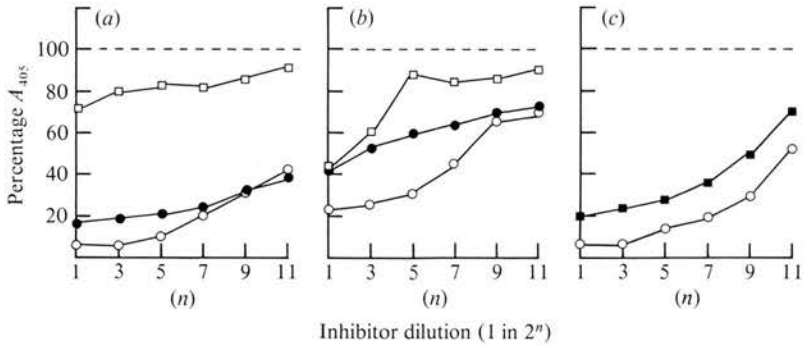

Fig. 7. Inhibition of ELISA reactions of GNAB 4 antiserum by untreated (○), heated (●), periodate treated (□) homologous OM complex and homologous LPS (■). (*a*) and (*c*), reactions with GNAB 86 OM complex; (*b*), reaction with GNAB 97 OM complex.

Inhibition of GNAB 4 antiserum reactions. Figure 7(*a*) shows the results obtained in the reactions with OM complex from GNAB 86. This trend occurred in the reactions with nine strains, including the homologous strain. The strong inhibition by heated antigens indicated that carbohydrates were the predominant antigens in ELISA. Only the reaction with GNAB 97 (Fig. 7*b*) differed, but still showed that the most important antigens were carbohydrate.

Inhibition of GNAB 4 antiserum was also carried out with the homologous LPS preparation, with untreated homologous OM complex providing a positive inhibition control. The results obtained in the reactions with strain GNAB 86 (Fig. 7*c*) indicated strong inhibition by LPS. A very similar picture was obtained in the reactions with five other strains. In the reactions with the strains GNAB 88, 97 and 98, inhibition by LPS was slightly less than in the GNAB 86 reaction, but was still very significant. Inhibition by LPS was weakest in the homologous reaction.

DISCUSSION

Throughout this study, an EDTA-45 °C-mild ultrasonication extract of whole cells was used as the OM complex antigen. From previous work (Poxton & Ip, 1981; Poxton *et al.*, 1982; Kasper & Seiler, 1975), we know that this type of preparation represents the outer membrane and associated molecules, including capsule, and contains species-specific antigens.

The techniques of CIE and CLIE have been useful in showing the array of antigens that exist at the cell surface of *B. fragilis*. By these methods, we have detected certain cross-reactions between strains and have examined the antigens present in extracted LPS in relation to OM complex antigens. Most previous studies of the cross-reacting precipitating antigens of *B. fragilis* have used immunodiffusion methods. Immunodiffusion has been applied to the analysis of various extracts of *B. fragilis* and has shown complex patterns of cross-reaction (Abshire *et al.*, 1979; Cherniak *et al.*, 1979; Meisel-Mikolajczyk *et al.*, 1981). It had been concluded that considerable antigenic heterogeneity exists between strains of *B. fragilis*. In the present study, although variations in cross-reacting patterns were observed, two of the test antisera (NCTC 9344 and GNAB 92) each detected heat labile, precipitating antigens common to all strains tested. Such heat labile common precipitating antigens have not previously been reported but Schwan *et al.* (1981) have demonstrated a heat labile surface antigen associated with non-homogeneous immunofluorescence staining. The reactions of the GNAB 4 antiserum were similar to those observed by previous workers in that no common antigen was demonstrated, heterologous strains cross-reacted with any one of three carbohydrate antigens and no cross-reactions could be demonstrated in some strains. The reason that GNAB 4 antiserum did not detect the single common antigen may have been because this organism has a well defined large capsule which masked, or was dominant over, the common antigen. This is

supported by the CIE results with GNAB 4 antiserum and homologous LPS and EDTA preparations where the major antigens in both preparations were found to be heat stable and periodate labile. We know from other studies (unpublished results) that the LPS preparation of GNAB 4 contains much high molecular weight capsular polysaccharide.

The nature of the heat labile, common, precipitating antigen(s) detected by the antisera to NCTC 9344 and GNAB 92 strains is not yet fully characterized, but attempts are being made to purify it. Both these strains apparently express this antigen when injected into rabbits. They do not exhibit obvious capsules.

An ELISA has been used to detect largely species-specific reactions of *B. fragilis* antisera with OM complex from heterologous strains (Poxton, 1979). SDS-PAGE of OM complex has shown marked similarities in the polypeptide profiles of different *B. fragilis* strains (Poxton & Brown, 1979; Kasper & Seiler, 1975), suggesting that proteins could act as species-specific antigens in ELISA. Alternatively, the species-specific carbohydrate antigen(s) isolated from OM complex (Kasper & Seiler, 1975; Kasper, 1976) and aqueous phenol extracts (Kasper *et al.*, 1983) may be the major cross-reacting antigen(s) in ELISA. The nature of the antigens reacting in ELISA was investigated in the present study by ELISA inhibition. The results indicated that the predominant cross-reacting antigens were in most cases periodate sensitive and heat resistant and were therefore carbohydrates. The extent to which protein (heat labile, periodate resistant) antigens appeared to be involved in ELISA reactions depended on the individual cross-reacting strain. Although protein antigens were of some significance in ELISA for at least two cross-reacting strains (GNAB 88 and GNAB 97), carbohydrate antigens still contributed to these reactions.

There was clearly limited correlation between the CLIE and ELISA results: in the reactions of GNAB 4 antiserum, heat stable carbohydrates were the important cross-reacting antigens in both systems; however, CLIE did not detect cross-reactions with certain strains that cross-reacted strongly in ELISA. Heat stable carbohydrates were also predominant in the ELISA reactions with GNAB 92 antiserum, yet CLIE detected only a single, heat labile, cross-reacting antigen with this antiserum. The limited correlation between the two techniques is not unexpected considering the far greater sensitivity of ELISA over CLIE. Also, CLIE only detects precipitating antigens, while ELISA will detect monovalent antigens.

It is not clear which of the carbohydrate components of the OM complex were reacting in ELISA. Inhibition of ELISA reactions of GNAB 4 antiserum with homologous LPS indicated that LPS contained most of the cross-reactive antigens. The nature of the antigens present in the LPS preparations is currently under investigation in this laboratory.

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Immunochemistry of the Surface Carbohydrate Antigens of *Bacteroides fragilis* and Definition of a Common Antigen

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The components extracted by aqueous phenol from whole cells of *Bacteroides fragilis* were analysed by SDS-PAGE and immunoblotting and shown to consist of a series of strain-specific, cross-reactive and common antigens. Regularly-spaced ladder patterns on silver-stained gels indicated that in most strains the LPS was present as a predominantly smooth type, but with chain lengths of varying molecular mass, ranging within each particular strain from essentially rough forms to long chain-length smooth forms. The rough form of the LPS at the gel front possessed an antigen common to most of the strains investigated. Another antigen, which migrated behind the rough LPS on SDS gels, was common to all strains of the species. The smooth LPS forms and the other high molecular mass components were strain-specific antigens. Previously published methods are not capable of producing pure LPS or capsular polysaccharide for this organism.

INTRODUCTION

Bacteroides fragilis, the most frequently isolated anaerobic bacterium from clinical specimens, appears to have a greater pathogenic potential than its close relatives, e.g. *B. vulgatus* and other members of the *B. fragilis* group. Surface structures have been associated with this virulence (Onderdonk *et al.*, 1977). Much has been published on the chemistry and immunochemistry of structural components but confusion exists. Many workers (e.g. Kasper *et al.*, 1983) assume that the surface carbohydrates of this Gram-negative bacterium consist simply of lipopolysaccharide (LPS) and 'capsular polysaccharide'. Hofstad (1981) and Cousland & Poxton (1984), however, have demonstrated a complex set of surface carbohydrates. Much of the apparent confusion stems from the difficulty in preparing pure surface components. Kasper *et al.* (1983) and Weintraub *et al.* (1985) claimed to have developed methods for the preparation of pure capsular polysaccharide free from LPS, and of LPS free from capsular polysaccharide, protein and nucleic acid. Starting with an aqueous phenol extract of whole cells, capsular polysaccharide was prepared by gel filtration in the presence of a detergent, whereas LPS was prepared by further extraction with a phenol/chloroform/petroleum spirit mixture. The authors suggested that the LPS of *B. fragilis* is a rough-type molecule and is both chemically and antigenically similar in all the strains investigated.

The development of a method for demonstrating the heterogeneity of LPS on polyacrylamide gels (Tsai & Frasch, 1982) has been applied to many species of Gram-negative bacteria. In a preliminary report (Cousland & Poxton, 1983), we showed that *B. fragilis* possesses a complex mixture of aqueous phenol-extractable surface carbohydrate antigens. A series of closely spaced bands in a ladder pattern on the silver-stained polyacrylamide gel was reminiscent of the pattern produced by the smooth form of LPS from other bacteria. This led us to suggest that *B. fragilis* possesses smooth type LPS. We also showed that a common antigen ran at the gel front and was associated in some unknown way with the rough form of the molecule.

In the present paper we extend our earlier work by studying aqueous phenol extracts of a range of *B. fragilis* strains with a combination of SDS-PAGE and immunoblotting. We report on the complexity of the carbohydrates of the cell-surface of *B. fragilis* and we define the common antigen.

METHODS

Bacterial strains and growth conditions. B. fragilis strains NCTC 9343 and 9344 were obtained from the National Collection of Type Cultures, Colindale Avenue, London, UK. Clinical strains of *B. fragilis*, GNAB 4, 82, 85, 90 and 92, were from our laboratory collection.

All strains were cultured in 2 to 6 litre batches of proteose peptone/yeast extract (PPY) medium (Deacon *et al.*, 1978) statically for 18 h in an anaerobic atmosphere (80% N_2, 10% CO_2 and 10% H_2) at 37 °C.

Preparation of aqueous phenol extract. Lyophilized bacteria were resuspended to a concentration of approximately 5% (w/v) in distilled water and heated to 67 °C. An equal volume of 90% (w/w) aqueous phenol at 67 °C was added to the suspension and the mixture was stirred for 15 min at 67 °C. After cooling to 4 °C the phases were separated by centrifugation at 10000 ***g*** for 10 min. The upper aqueous phase was dialysed against running water for 18 h, concentrated to approximately 20% of the original volume by rotary evaporation and centrifuged at 100000 ***g*** for 3 h to sediment the insoluble fraction. This was washed once by suspension in water with the aid of a syringe fitted with a no. 26 gauge needle, and recentrifugation. The pellet was finally resuspended in water and lyophilized.

Fractionation of aqueous phenol extract. The lyophilized pellet from the aqueous phenol extract (5 mg) was solubilized in deoxycholate buffer and fractionated on a column (55 × 1·6 cm) of Sephacryl S-300 by the method of Kasper *et al.* (1983). Fractions (50 × 2 ml) were analysed for precipitating antigen by fused rocket immunoelectrophoresis in the presence of 1% Triton X-100 (Svendsen, 1973), and for total antigen by dot-blotting onto nitrocellulose membrane and reaction with antiserum to strains of *B. fragilis* as described below.

Preparation of rough LPS. Lyophilized, dialysed aqueous phase from the aqueous phenol extraction of *B. fragilis* NCTC 9344 was extracted with phenol/chloroform/petroleum by the method used for LPS extraction by Weintraub *et al.* (1985). The material remaining insoluble after phenol/chloroform/petroleum extraction was resuspended in distilled water, re-extracted with an equal volume of 90% aqueous phenol as described above and the aqueous phase dialysed and lyophilized.

Preparation of antisera. Antisera were raised in New Zealand White rabbits to whole, live, washed cells of *B. fragilis* strains NCTC 9344, GNAB 4 and GNAB 92 by the method of Poxton (1979).

Monospecific polyclonal antiserum was raised to the *B. fragilis* common antigen by a method briefly referred to in a Bio-Rad pamphlet (no. 48EG, 1984). The aqueous phenol extract (containing 112 μg carbohydrate) was separated as a band on SDS-PAGE and transferred to nitrocellulose as described below. This was done in duplicate. Guide strips were cut from the nitrocellulose and reacted with whole cell antiserum to locate the common antigen. Strips 2 mm wide containing the common antigen were cut from the unreacted nitrocellulose membranes. They were dried in a stream of compressed air, cut into small pieces and dissolved in dimethyl sulphoxide (100 μl cm^{-2}). This was mixed with an equal volume of Freund's complete adjuvant (Difco) and injected subcutaneously in approximate 0·1 ml volumes into several sites on the back of a rabbit. This procedure was repeated four times but with Freund's incomplete adjuvant over a period of two months. Test bleedings taken at intervals during the injection schedule were screened by dot-blotting against homologous aqueous phenol extract. The antiserum reached its highest titre one month after the last injection.

SDS-PAGE. This was done on both 10% and 12% slab gels with the buffer system of Laemmli (1970) by the method described by Poxton & Brown (1979). Gels were oxidized with periodate and stained with silver by the method of Tsai & Frasch (1982).

Electrophoretic transfer and immunoblot. This was done by the method described by Cousland & Poxton (1983), with the Bio-Rad Immunoblot reagents, except that the nitrocellulose membrane was 0·2 μm pore size (Sartorius). Antigens were either electrophoretically transferred, or applied directly in 1 μl volumes as dots for dot-blotting. The antiserum raised against whole cells was used at a dilution of 1 in 250 while that raised against the common antigen was used at a dilution of 1 in 40.

Analytical methods. Carbohydrate was measured by the method of Dubois *et al.* (1956) with glucose as a standard. Protein was measured by the Lowry method.

RESULTS

The bacteria grew well in PPY medium to yield 0·5–1·0 g dry weight l^{-1} after overnight incubation. After aqueous phenol extraction and ultracentrifugation, yields of extract were 0·4% to 0·9% of the bacterial dry weight. All strains were grown on more than one occasion; yields were similar and subsequent analyses were identical.

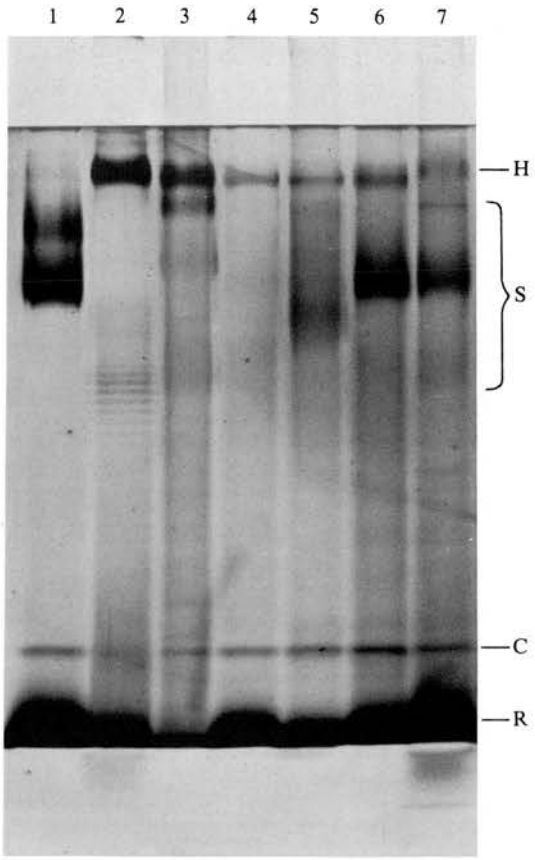

Fig. 1. Silver-stained SDS-polyacrylamide (12%) gels of aqueous phenol extracts (10 μg carbohydrate) of seven strains of *B. fragilis*. Track 1, NCTC 9343; track 2, NCTC 9344; track 3, GNAB 4; track 4, GNAB 82; track 5, GNAB 85; track 6, GNAB 90; track 7, GNAB 92. H, High molecular mass band; S, multi-banded pattern typical of smooth LPS; C, common antigen; R, rough LPS at gel front.

Samples of dry extract were resuspended in distilled water to a concentration of 5 mg ml^{-1} and assayed for carbohydrate and protein. There was a negligible amount of protein, and phenol/sulphuric acid positive carbohydrate (Dubois *et al.*, 1956) accounted for about 20% only of the dry weight of the extract. No further chemical assays were done.

SDS-PAGE and immunoblotting of aqueous phenol extracts

Each extract was dissolved to a concentration of 400 μg carbohydrate ml^{-1} in the SDS/2-mercaptoethanol solubilization buffer of Laemmli (1970), and heated to 100 °C for 3 min. Samples (25 μl) were applied to a 12% SDS-polyacrylamide gel. Fig. 1 shows examples of patterns obtained after separation and silver staining of aqueous phenol extracts from seven strains of *B. fragilis*. In all tracks there is a multi-banded pattern (labelled S), of varying intensity, which is similar to smooth LPS of other Gram-negative bacteria. In some tracks (3, 4 and 5) the fine banding pattern is extremely faint and the general appearance is more like that of rough LPS. There is material of high molecular mass with a characteristic dumb-bell shape in most of the tracks (labelled H). A pronounced band (C) is present behind the main front band (R). No Coomassie-blue-staining material was observed.

Three separations identical to that shown in Fig. 1 were done, and the components from each were transferred to a sheet of nitrocellulose and probed with antisera raised to whole cells of

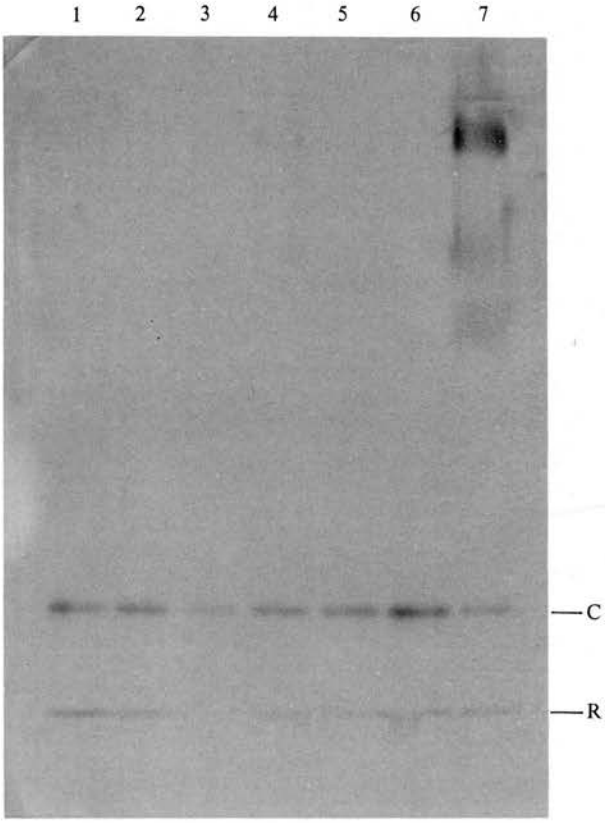

Fig. 2. Immunoblots from SDS-polyacrylamide (12%) gels of aqueous phenol extracts of the strains used in Fig. 1. The blots were probed with antiserum raised against whole cells of *B. fragilis* GNAB 92 and anti-rabbit horseradish peroxidase conjugate. See Fig. 1 for abbreviations.

three of the strains: NCTC 9344, GNAB 4 and GNAB 92. An example of the results (with GNAB 92 antiserum) is shown in Fig. 2. All strains have one antigen in common, a discrete band migrating behind the front (labelled C). The band migrating at the front (R) is an antigen common to all strains except GNAB 4, whereas antigens in the upper part of the gel were detected only in the homologous strain (track 7) and weakly in strain GNAB 82 (track 4). With the NCTC 9344 antiserum, a similar pattern was seen: an antigen common to all strains was detected just behind the gel front, a band at the gel front was common to all except GNAB 4 and a series of higher molecular mass bands were common to the homologous strain, NCTC 9343 and GNAB 4. With the GNAB 4 antiserum, the common band behind the gel front was apparent, and a series of higher molecular mass bands common to the same three strains as with the NCTC 9344 were visible, but the band at the front was specific only to the homologous reaction. Pre-immune sera gave no reactions.

Fractionation of aqueous phenol extract

As the aqueous phenol extract consisted of a series of different antigens demonstrable by SDS-PAGE and immunoblotting, the whole extract was subjected to gel filtration in the presence of deoxycholate by the method developed by Kasper *et al.* (1983) for the production of capsular polysaccharide free from LPS. The extract from *B. fragilis* GNAB 92 was solubilized and fractionated on Sephacryl S-300. Analysis of the fractions by fused rocket immunoelectrophoresis with homologous whole cell antiserum showed that only fractions eluted at the void volume of the column contained precipitating antigens. When the fractions were analysed by dot blotting,

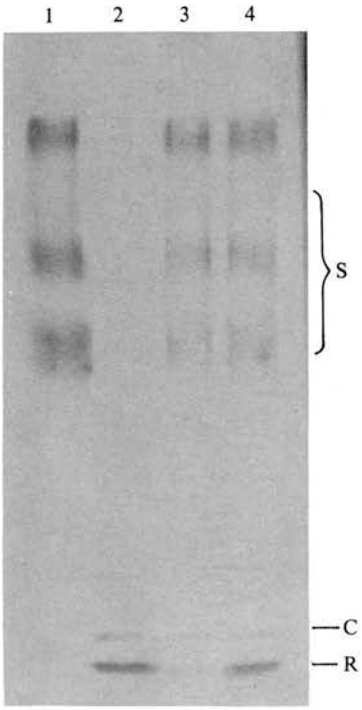

Fig. 3

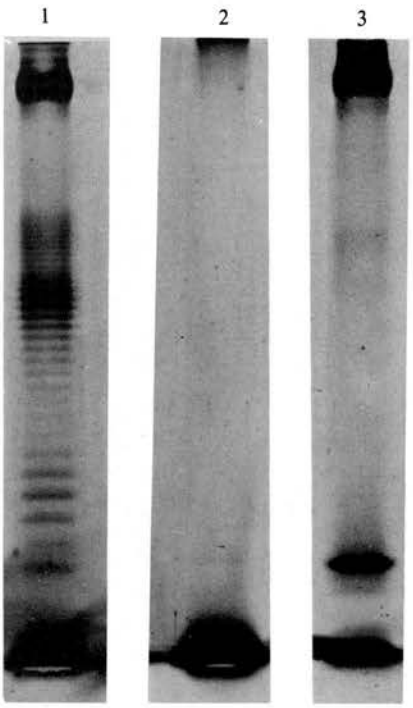

Fig. 4

Fig. 3. Immunoblots from SDS-polyacrylamide (10%) gels of an aqueous phenol extract of *B. fragilis* GNAB 92 after fractionation on Sephacryl S-300. Track 1, pooled void volume antigenic fractions; track 2, pooled included antigenic fractions; track 3, fractions from between two antigenic peaks; track 4, unfractionated aqueous phenol extract. All tracks contained 10 μg carbohydrate. The blots were probed with homologous whole cell antiserum and horseradish peroxidase conjugate. See Fig. 1 for abbreviations.

Fig. 4. Silver-stained SDS-polyacrylamide (12%) gels of extracts (10 μg carbohydrate) of *B. fragilis* NCTC 9344. Track 1, aqueous phenol extract; track 2, phenol/chloroform/petroleum extract of aqueous phenol extract; track 3, material remaining insoluble after phenol/chloroform/petroleum extraction.

two groups of antigen-containing fractions were detected, one corresponding to the column void volume which was the same as that detected by fused rocket immunoelectrophoresis and one containing lower molecular mass material. With the two other antisera (GNAB 4 and NCTC 9344), only the lower molecular mass material reacted. These findings indicate that the first peak contained strain-specific antigens while the second contained cross-reactive antigens.

The fractions that corresponded to each of the two peaks of antigen as demonstrated by dot blotting, and the fractions between the two peaks, were combined into three pools, lyophilized and examined by SDS-PAGE and immunoblotting; both techniques gave identical results. The patterns produced with homologous antiserum are shown in Fig. 3. The void volume fractions (track 1) contained a series of antigens of which the central group (labelled S), on close examination of the original, consisted of a series of discrete fine bands. The included fractions (track 2) produced only two bands corresponding to the common *B. fragilis* antigen (C), and the front band (R). The pooled fractions between the peaks (track 3) contained a mixture of the antigens in both of the peaks. Track 4 is of the aqueous phenol extract before fractionation; fractionation of the aqueous phenol extract of GNAB 4 gave similar results.

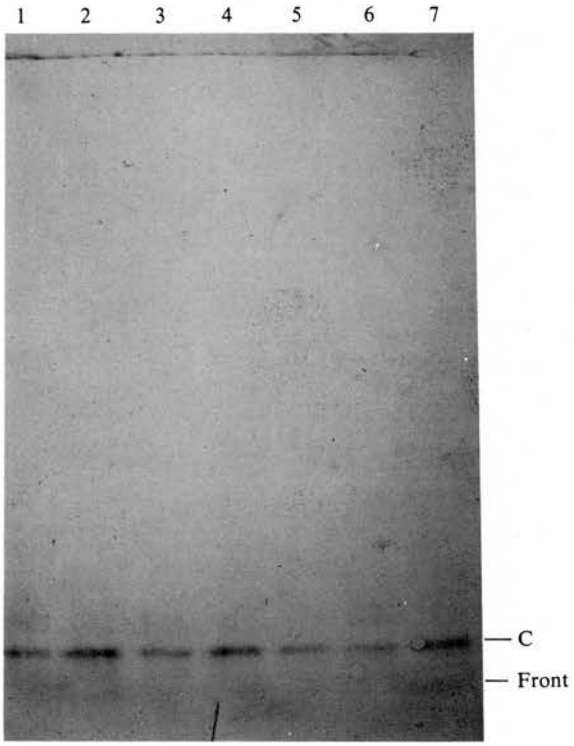

Fig. 5. Immunoblots from SDS-polyacrylamide (12%) gels of aqueous phenol extracts of the strains used in Fig. 1. The blots were probed with monospecific antiserum raised against the common antigen excised from nitrocellulose.

Comparison of rough LPS with aqueous phenol extract

The pattern produced on SDS-PAGE of the phenol/chloroform/petroleum-extracted fraction of the aqueous phenol extract of NCTC 9344, i.e. the fraction corresponding to the LPS studied by Weintraub *et al.* (1985), was compared with that of the aqueous phenol extract and that of the material that remained insoluble after the phenol/chloroform/petroleum treatment (Fig. 4). A ladder pattern typical of smooth LPS was found with the aqueous phenol extract, with the dumb-bell shaped high molecular mass band. Track 2 shows the PCP fraction, which was typical of rough LPS, with only a single heavily staining band at the front. Track 3, containing the insoluble material remaining after PCP treatment, had a strongly staining dumb-bell shaped band at the top, a faintly staining ladder pattern that became stronger after longer development, an intensely stained band running behind the front (subsequently shown by blotting to be the common antigen) and also a band at the dye front.

Demonstration of the antigenic specificity of the B. fragilis common antigen

Common antigen, purified by SDS-PAGE, immunoblotting and extraction from nitrocellulose membranes, was injected into a rabbit to raise monospecific antiserum. In immunoblots against aqueous phenol extracts prepared from the seven strains used in Figs 1 and 2, the serum reacted strongly with the common band of the *B. fragilis* strains (Fig. 5).

DISCUSSION

In this study we have shown that aqueous phenol extracts of *B. fragilis* contain a series of antigens that can be detected by silver-staining and immunoblotting. Most strains possess antigens that include (i) a rough type LPS which runs at the gel front, (ii) a common antigen which runs behind the front, (iii) a smooth LPS which runs as a series of closely spaced bands,

and (iv) one other antigen, with a high molecular mass and with a characteristic dumb-bell pattern, which we suggest is the capsular polysaccharide.

The recent studies of Weintraub *et al.* (1985) on the chemistry and immunochemistry of the LPS of *B. fragilis* were based on the assumption (shown here to be false) that the species produced only a rough form of LPS; the system used selectively extracted rough LPS and the fraction that would have contained the smooth form was discarded. In the earlier studies of these workers (Kasper *et al.*, 1983), the Sephacryl S-300 chromatography separated the aqueous phenol extract into two major fractions; the higher molecular mass fraction consisted of 'capsular polysaccharide', while the second fraction contained LPS. In our studies, the first peak consisted of smooth LPS together with another high molecular mass antigen, probably capsular polysaccharide, and the second peak contained rough LPS and the common antigen.

The nature of the common antigen is not known. The monospecific antiserum raised against it shows that it is antigenically distinct from the rough form of the LPS, although in some immunoblots a weak reaction was seen with some of the front material. Studies are currently in progress to purify and characterize the antigen with monoclonal antibodies, but at present we can only speculate on its nature. It may either be totally distinct from the LPS and be analogous to the enterobacterial common antigen (Mannel & Mayer, 1978), as it is purified in a similar way, or be related to the rough LPS but with a unique and dominant epitope: it could be lipid A plus a complete core – the unique epitope, distal from the lipid end, being normally substituted with O antigen. The material that runs at the gel front, which is common to all but one of the strains of *B. fragilis* that we have investigated, may be lipid A with an incomplete core. Its epitopes would be expressed along the linear core portion of all or most LPS molecules. The smooth LPS and the high molecular mass 'capsular' antigen are not common antigens. There is a certain amount of cross-reaction between the strains of the species but there appear to be type-specific antigens which could be used in serotyping.

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Immunochemistry of the cell surfaces of *Bacteroides bivius* and *Bacteroides disiens*

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Summary. Outer membranes were extracted from seven strains of *Bacteroides bivius* and six strains of *B. disiens* by the Sarkosyl method. Lipopolysaccharides (LPS) were extracted from the same strains by the Proteinase K method, and from three strains of each species by an aqueous phenol method. Analysis of the outer-membrane proteins by SDS-PAGE demonstrated that, within a species, very similar patterns with many shared or common bands were produced, but there were sufficient differences between species to allow separation. Immunoblotting with antisera raised against whole cells of each of the type strains showed that many antigens were shared between species. Smooth LPS was present in both species. By immunoblotting, the O-antigen of *B. disiens* was shown to be common to all six strains, and there was no cross-reaction between the *B. disiens* antiserum and *B. bivius* LPS. The O-antigen of *B. bivius* was not detected by immunoblotting with homologous antiserum, but antiserum to *B. bivius* reacted with a series of common low molecular mass antigens that were present in LPS preparations from strains of both species.

Introduction

Bacteroides bivius and *B. disiens* are closely related species of anaerobic, gram-negative bacteria that share the same main ecological niche, being most commonly isolated from the vagina and cervix in both health and disease and from genital infections in the male (Holdeman and Johnson, 1977; Duerden, 1980). Both species are non-pigmented, bile sensitive, weakly saccharolytic and proteolytic, and do not produce indole. They are differentiated by the ability of most *B. bivius* strains to produce acid from lactose and galactose whereas no strains of *B. disiens* ferment either of these sugars. DNA hybridisation has shown that they are distinct species (Holdeman *et al.*, 1977; Holdeman and Johnson, 1977).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell-surface proteins has been a useful aid in the identification of species and sub-types of a range of bacteria, including anaerobes (Poxton and Brown, 1979; Moore *et al.*, 1980). The aim of the present study was to use SDS-PAGE in conjunction with immunoblotting to compare the outer-membrane proteins and lipopolysaccharide (LPS) of several strains of *B. bivius* and *B. disiens* to determine any inter- or intra-species similarities or differences. The Sarkosyl method for the preparation of outer-membrane proteins and the Proteinase K method for LPS were chosen for their simplicity and applicability to small culture volumes; these advantages make the methods particularly useful for diagnostic or taxonomic investigations. LPS extracted by the Proteinase K method was compared with that extracted by the classical aqueous phenol method.

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Materials and methods

Bacterial strains

B. bivius strains VPI 6822 and VPI 6318, and *B. disiens* strains VPI 8057 and VPI 7582 were obtained from the Virginia Polytechnic Institute and State University, Blacksburg, VA, USA. Strain GNAB 12 was isolated from a high vaginal swab in this department. The MW series of strains was supplied by Dr B. Watt, City Hospital, Edinburgh. Identity of strains was confirmed by the method of Brown *et al.* (1989). Tests included bile tolerance, indole production, fermentation of glucose, lactose, sucrose and maltose, and gelatinase activity.

Preparation of outer membrane

Cultures were prepared by seeding 500-ml volumes of pre-reduced proteose peptone-yeast extract (PPY) broth (Deacon *et al.*, 1978) with 25 ml of 48-h starter cultures

in the same medium. They were incubated anaerobically (H_2 90%, CO_2 10%) for 24 h at 37°C. The cells were harvested and washed once in 200 ml of phosphate-buffered saline (50 mM phosphate buffer, pH 7·4, containing 0·15 M NaCl; PBS). The centrifuge tubes were drained and the pellet was suspended in 5 ml of distilled water. The cells were broken by passage through a French pressure cell (Aminco, Silver Springs, MD, USA) at 6000 psi (42 MPa) at 0°C. Unbroken cells were removed by centrifugation at 6000 *g* for 10 min at 4°C. A modification of the method of Filip *et al.* (1973) was used for Sarkosyl treatment. One volume of a 7% w/v solution of sodium N-lauroyl sarcosinate was added to nine volumes of the broken-cell suspension. The insoluble outer membrane was harvested and washed once in 5 ml of distilled water by centrifugation at 50 000 *g* at 4°C for 1 h. The pellet was resuspended in 0·5 ml of distilled water by repeated aspiration into a syringe through a 26-gauge needle. The suspension was stored at −20°C. Protein was assayed by the method of Lowry *et al.* (1951).

Preparation of LPS

The Proteinase K method for LPS extraction was modified from Hitchcock and Brown (1983). Volumes (5 ml) of the cultures prepared for Sarkosyl extraction were harvested and washed twice in PBS by centrifugation at 4000 *g* at 4°C for 15 min. The pellet was suspended in PBS to an A_{525} of 0·6 and 1·5-ml volumes were centrifuged at approximately 10 000 *g* for 90 s (Microfuge Beckman Model B). The pellet was solubilised in 50 μl of PAGE sample buffer (0·0625 M Tris-HCl, pH 6·8, containing sodium dodecyl sulphate 2% w/v, glycerol 10% v/v, 2-mercaptoethanol 1% v/v and bromophenol blue 0·001% w/v) and heated in a boiling water-bath for 10 min. Proteinase K (25 μg) (Sigma Protease XI) in 10 μl of sample buffer was added to each tube and then incubated at 60°C for 1 h. Samples were stored at −20°C.

The aqueous phenol method for the extraction of LPS was that of Poxton and Brown (1986).

Carbohydrate was assayed by the method of Dubois *et al.* (1956).

SDS-PAGE

SDS-PAGE was performed on 10% slab gels with the Laemmli buffer system (Laemmli, 1970) by the method of Poxton and Brown (1979). Samples (25 μl or 100 μl) of the Proteinase K LPS extracts, 50-μl or 100-μl samples of the aqueous phenol extracts containing 10 μg or 20 μg of carbohydrate respectively, or 50-μl samples containing 9–25 μg of protein of the Sarkosyl preparations were loaded on to the gels. The LPS separating gels were stained by a modification of the silver stain methods of Tsai and Frasch (1982) and Hitchcock and Brown (1983). The gel was fixed in 200 ml of propan-2-ol 25% v/v, acetic acid 7% v/v overnight, and then oxidised in freshly prepared periodic acid 1·05 g in 150 ml of distilled water containing 4 ml of the above fixative for 5 min. The gel was then washed in at least four changes of 200 ml of distilled water over 4 h, drained and added to fresh ammoniacal silver nitrate solution—1·4 ml of ammonia solution (SG 0·88) was added to 21 ml of NaOH 0·36%, and 4 ml of $AgNO_3$ 19·4% solution was added with vigorous agitation; after the brown precipitate had disappeared, the volume was made up to 100 ml with distilled water. After 15 min, the gel was washed in at least four changes of distilled water over 40 min and then transferred to a fresh solution of citric acid 0·005% in 200 ml of formaldehyde 0·019% at 25°C. When the desired staining intensity was reached, the gel was washed repeatedly in large volumes of distilled water.

The Sarkosyl outer-membrane gels were stained with Coomassie blue by the method of Poxton and Sutherland (1976).

Immunoblot transfer

This was based on the method of Towbin *et al.* (1979) as described by Cousland and Poxton (1983) with BioRad immunoblotting reagents, but the nitrocellulose membrane was of 0·2-μm pore size (Sartorius). For dot blotting, 2 μl of aqueous phenol-extracted LPS was applied directly to the nitrocellulose. Antisera were used at a dilution of 1 in 100.

Preparation of antisera

Antisera were raised in rabbits against washed whole cells of the type strains—*B. bivius* VPI 6822 and *B. disiens* VPI 8057—and against *B. bivius* VPI 6318 by the method of Poxton *et al.* (1982).

Samples of the antisera raised against the type cultures were absorbed with their homologous aqueous phenol-extracted LPS. Serum (2 ml) was mixed with 1 ml of a solution containing 1 mg of LPS (0·2 ml of an aqueous solution of LPS 5 mg/ml w/v + 0·8 ml of PBS) and agitated on a blood-cell mixer at 15°C for 1·5 h. After centrifugation at 10 000 *g* at 15°C for 5 min, the supernate was re-extracted as above. Unreacted LPS and immune complexes were removed by two cycles of centrifugation at 100 000 *g* at 4°C for 3 h, and the supernate was collected and stored at −20°C. The absorbed sera were shown by dot blotting not to react at a final dilution of 1 in 100 with their homologous aqueous phenol LPS.

Results

SDS-PAGE and immunoblotting of Sarkosyl outer-membrane preparations

Fig. 1 shows examples of the patterns obtained after separation and Coomassie-blue staining of Sarkosyl extracts of seven strains of *B. bivius* and six of *B. disiens*. The patterns produced by the *B.*

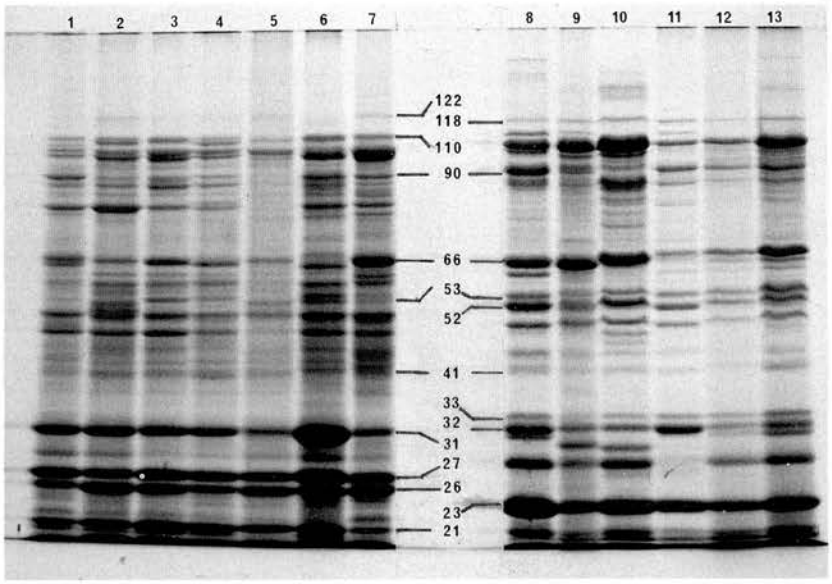

Fig. 1. SDS-PAGE of Sarkosyl outer-membrane preparations of *B. bivius* and *B. disiens* strains separated on acrylamide 10% slab gels stained with Coomassie blue. Track 1, *B. bivius* VPI 6822; 2, MW 1; 3, MW 3; 4, MW 6; 5, MW 8; 6, MW 15; 7, VPI 6318; 8, *B. disiens* VPI 8057; 9, MW 20; 10, VPI 7582; 11, GNAB 12; 12, MW 19; 13, MW 21. Molecular masses are in Kda.

bivius strains (tracks 1–7) show much similarity to one another with many shared or common bands and only a few strain-specific bands, and have in particular four major common bands of approximately 31, 27, 26 and 21 Kda. The patterns produced by the six *B. disiens* strains (tracks 8–13) show less similarity to each other with more strain-specific bands but still have a great many shared or common bands. One major common band of 23 Kda, and a common duplex of 32–33 Kda are seen. Several bands appeared to be shared by both species, and bands of approximately 90, 66, 53 and 41 Kda appear to be common to all strains of both species.

Three gels identical to those shown in fig. 1 were run and each was transferred to nitrocellulose membranes. One of these blots was probed with antiserum raised against whole cells of *B. bivius* VPI 6822 (fig. 2a) and the second was probed with antiserum to *B. disiens* VPI 8057 (fig. 2b). The third blot was cut to separate the two species and each was probed with its homologous antiserum which had been absorbed with its own LPS. Fig. 2a shows that all the *B. bivius* strains (tracks 1–7) have almost identical profiles except for slight quantitative differences and a few strain-specific antigens. There are many common antigens and the general profile bears some resemblance to the pattern in fig. 1, all the major protein bands being antigenic. A greatly elongated high-molecular-mass band partially obscuring the separated protein bands and clearly seen in the upper half of the homologous reaction (track 1) and faintly in track 2 were also seen in immunoblots of Proteinase K extracts of the same two strains (see fig. 4a later). Curved bands seen clearly at the front of tracks 1, 3 and 6, and less clearly in tracks 2, 4, 5 and 7, were not seen in the Coomassie blue-stained gel. When these seven Sarkosyl preparations were probed with LPS-absorbed *B. bivius* antiserum, the latter two described bands were not seen, indicating that they were LPS antigens. Antigens of 53, 46 and 29 Kda were also not detected with the absorbed serum.

The heterologous reactions in tracks 8–13 show that there are a considerable number of *B. disiens* antigens that cross-react with *B. bivius* antiserum. Again, some of these antigens correspond to the *B. disiens* outer-membrane-protein bands in fig. 1. A few of the bands (122, 72 and 27 Kda) appear to be common to *B. bivius* and *B. disiens* but three of the four major common protein antigens of *B. bivius* appear to be species specific. The 23- and 33-Kda common protein bands were detected by the *B. bivius* antiserum.

Fig. 2b shows the same strains as in fig. 1, probed with the antiserum raised against *B. disiens* VPI 8057. The six *B. disiens* strains (tracks 8–13) share common antigens of 122, 118, 72, 52 and 33 Kda.

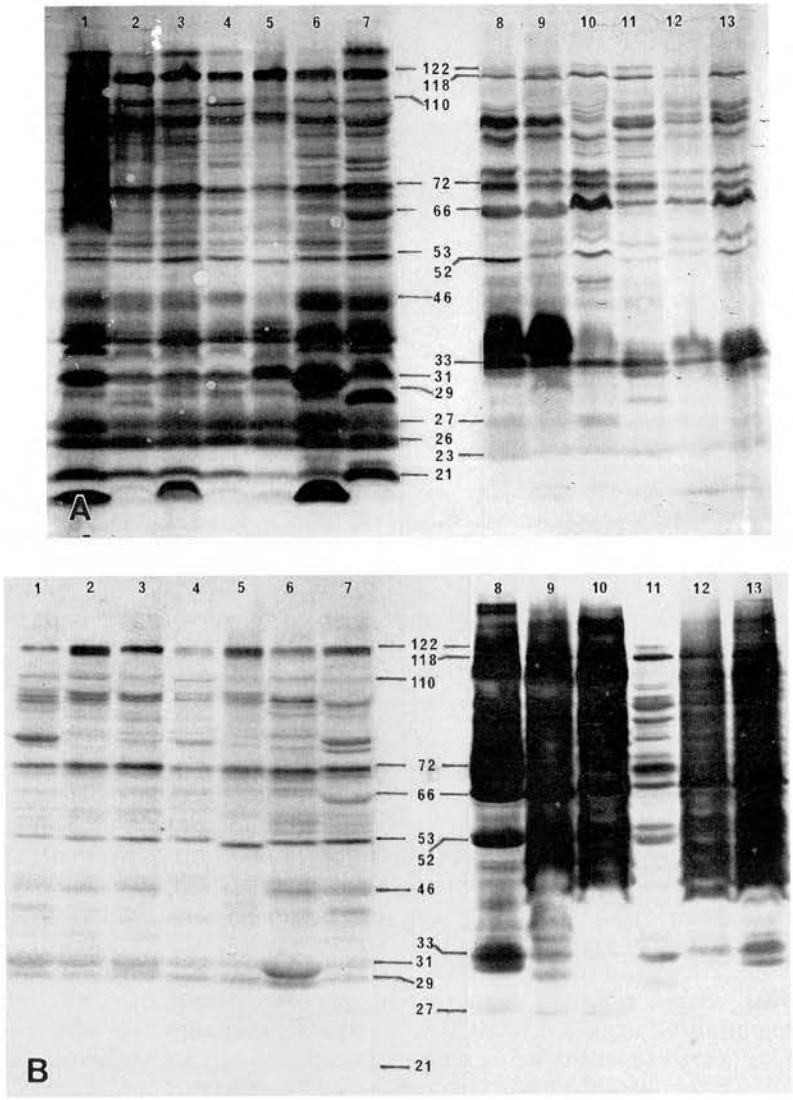

Fig. 2. Immunoblot of Sarkosyl outer-membrane preparations of *B. bivius* and *B. disiens* after separation as in fig. 1 and transfer to nitrocellulose membrane. Tracks were loaded with the same samples as in fig. 1. Gels were probed with (**a**) *B. bivius* VPI 6822 and (**b**) with *B. disiens* VPI 8057 whole cell antisera, followed by anti-rabbit IgG-horseradish peroxidase conjugate and colour development. Molecular masses are in Kda.

Several antigenic bands, seen most clearly in track 11, of 118, 52 and 33 Kda correspond to the protein bands in fig. 1. In the upper half of the other tracks, several extra bands, not revealed by the Coomassie blue-stained gel, have largely obscured the protein bands. These are shown to be LPS antigens below. When these six Sarkosyl preparations were probed with LPS-absorbed *B. disiens* antiserum, all tracks, with the exception of a few strain-specific bands, were identical to each other and to the pattern produced by strain GNAB 12 (track 11) in fig. 2b. Some of the low-molecular-mass protein bands, including the major 23-Kda common protein, are not apparently antigenic.

The heterologous reactions (tracks 1–7) again reveal a considerable number of cross-reacting antigens that are identical to the corresponding tracks in fig. 2a. However, in fig. 2b many of the bands in the lower half of the gel are either absent or gave very weak reactions. The elongated high-molecular-mass band seen in tracks 1 and 2 in fig. 2a were absent in fig. 2b.

SDS-PAGE and immunoblotting of aqueous phenol and Proteinase K LPS extracts

Fig. 3 shows examples of patterns obtained after separation and silver staining of aqueous phenol extracts of three strains of *B. bivius* and two of *B. disiens*. With the exception of *B. bivius* strain MW8 (track 3) even when overloaded, smooth LPS ladder patterns, resembling those found in some Enterobacteriaceae, were seen. A considerable amount of material was seen at the front of all tracks. The *B. bivius* tracks had in common a series of four or so low-molecular-mass bands which may be a low-molecular-mass ladder pattern, and an elongated high-molecular-mass band. The *B. disiens* tracks had two common bands in addition to the ladder pattern. The first was a high-molecular-mass dumbbell shaped band, and the other was a low-molecular-mass band (both arrowed).

When *B. bivius* aqueous phenol extracts were probed with antisera raised against *B. bivius* strains VPI 6822 and VPI 6318, no smooth LPS ladder pattern was seen. All other bands seen in the silver-stained gel were antigenic; at least three of the series of four low-molecular-mass bands were common antigens and the front material and elongated high-molecular-mass material appeared to be strain specific. Ladder patterns common to all three *B. disiens* aqueous phenol extracts were demonstrated when probed with antiserum raised against *B. disiens* VPI 8057 (data not shown). These results are identical to those described below for Proteinase K extracted LPS (see fig. 4a and b).

Proteinase K extracts of all strains belonging to both species were separated by SDS-PAGE, transferred to nitrocellulose and probed with homologous and heterologous antisera. With *B. bivius* VPI 6822 antiserum (fig. 4a), all seven extracts of *B. bivius* (tracks 1–7) showed reactions with the series of low-molecular-mass LPS antigens. An elongated high-molecular-mass band in tracks 1 and 2, and an antigen at the front in tracks 1, 3 and 6 correspond to antigens seen in the immunoblot of the Sarkosyl extracts of the same strains probed with the same serum (fig. 2a). Marked cross-reactions are seen with the series of low-molecular-mass antigens from five of the six Proteinase K extracts of *B. disiens* (tracks 8–13); strain GNAB 12 (track 11) showed no heterologous reaction. Faint high-molecular-mass smooth LPS ladder patterns are visible in tracks 8 and 10 (VPI 8057 and VPI 7582 respectively).

In the reaction between *B. disiens* Proteinase K extracts and homologous antiserum (fig. 4b), all six strains were seen to have a smooth LPS ladder pattern (tracks 8–13); the bands in track 11 (GNAB 12) were fainter and more widely spaced than in the other tracks. The low-molecular-mass bands that cross reacted strongly with the *B. bivius* antiserum have reacted only weakly with homolo-

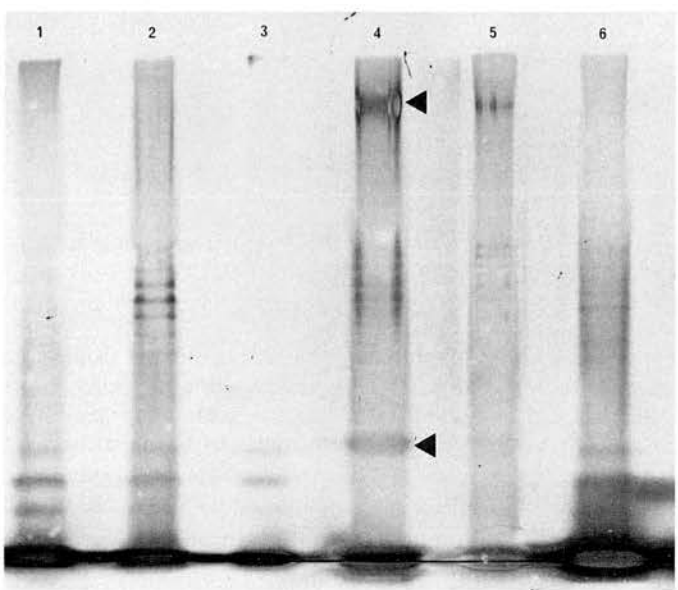

Fig. 3. SDS-PAGE of aqueous phenol extracts of *B. bivius* and *B. disiens* strains stained with silver. Tracks 1 and 6, *B. bivius* VPI 6822; 2, VPI 6318; 3, MW 8; 4, *B. disiens* VPI 8057; 5, VPI 7582. Tracks 1–5 were loaded with 10 μg of carbohydrate. Track 6 was loaded with 20 μg to demonstrate ladder pattern.

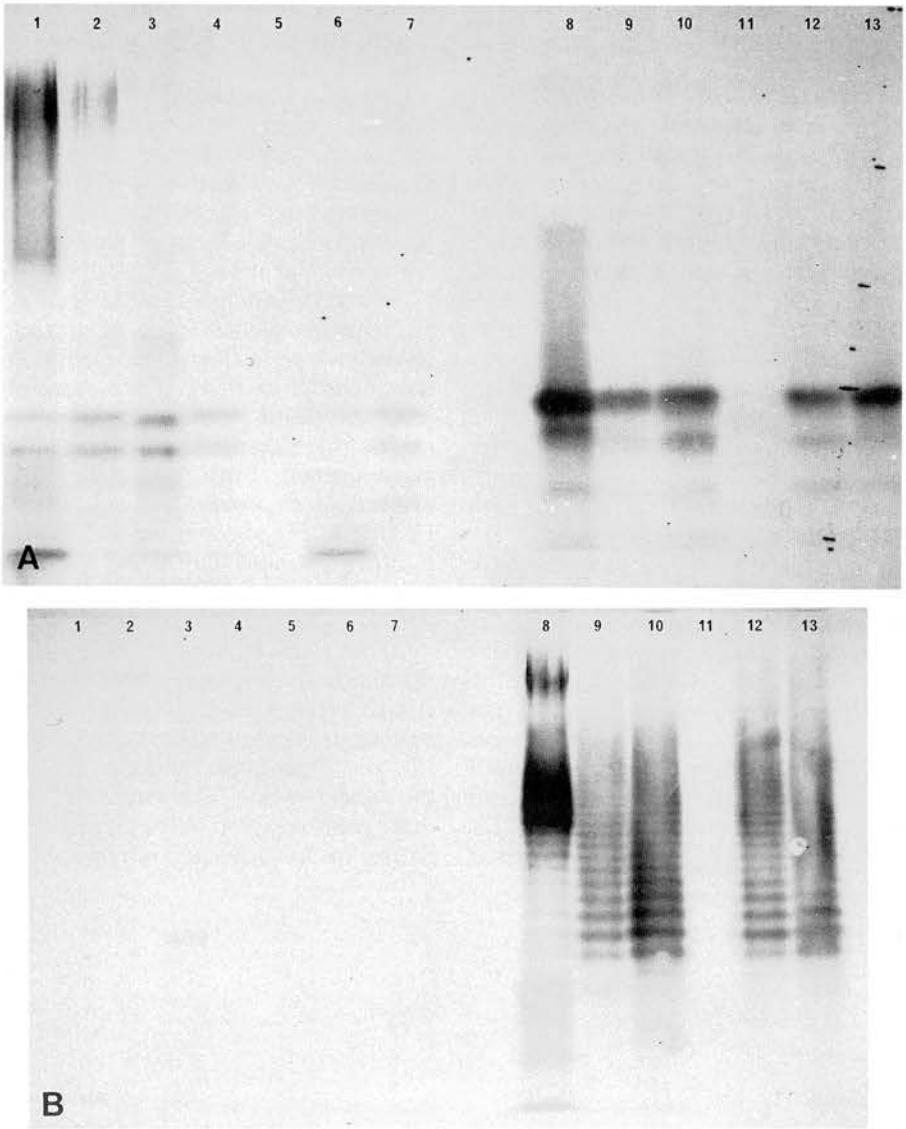

Fig. 4. Immunoblots of Proteinase K extracts of *B. bivius* and *B. disiens* strains, arranged as in fig. 1, probed with (**a**) *B. bivius* VPI 6822 and (**b**) *B. disiens* VPI 8057 whole cell antisera.

gous serum. A strain-specific dumb-bell-shaped antigen is seen near the top of track 8. The *B. bivius* Proteinase K extracts have not cross-reacted with the *B. disiens* antiserum.

Discussion

The Sarkosyl method for the preparation of outer membranes, when applied to *B. bivius* and *B. disiens*, gives protein patterns on SDS-PAGE that are similar to those produced by most gram-negative bacteria. Many different bands of protein are visualised and there are a few strongly staining bands which are usually classified as the major outer-membrane proteins. The patterns produced by strains belonging to the same species are sufficiently similar to each other but different from other species to allow identification to species level. Gram-negative bacteria that are closely related have cross-reactive outer-membrane antigens (Hofstra and Dankert, 1980) and these two closely related *Bacteroides* spp. have several protein antigens in common.

Immunoblotting of LPS with two *B. bivius* antisera detected a series of low-molecular-mass antigens that were common to that species and that also cross-reacted with a similar series of antigens of slightly different molecular mass in strains of *B. disiens*. These *B. bivius* antisera reacted with their homologous high-molecular-mass antigens but no clear ladder pattern was revealed. They did however, react weakly with high-molecular-mass ladder patterns in two strains of *B. disiens*. Antiserum raised against the type strain of *B. disiens* detected smooth LPS ladder patterns in all strains of that species, but did not cross-react with *B. bivius* LPS.

The LPS of gram-negative bacteria, especially the O antigenic part, is usually considered to be highly variable within a species. The core, however, is often much more conserved and there are regions, especially in the inner core, that are common within a species and even among species. This may explain the shared series of low-molecular-mass LPS antigens of these two species which consist of LPS molecules with a relatively higher proportion of core oligosaccharide than O antigen repeating units. The *B. bivius* strains appear to have produced a strong anti-core antibody response, whereas the *B. disiens* has produced a strong anti-O response.

In summary, *B. bivius* and *B. disiens* are closely related in respect to outer-membrane proteins although differences permit species identification. The two species are related serologically (Poxton *et al.*, 1982) and we have demonstrated in this study that cross-reactivity is due to shared outer-membrane proteins and low-molecular-mass LPS material, suggesting that they have common core structures. In this study, both species were shown to have smooth LPS; the typical O-antigen ladder pattern of *B. disiens* reacted well with homologous antiserum and appeared to be common to all strains with the possible exception of GNAB 12. A high-molecular-mass ladder pattern of *B. bivius* was not detected by immunoblotting but a strain-specific high-molecular-mass antigen, not resolved into a ladder pattern, was observed.

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THE DETECTION OF ANTIBODIES IN THE GINGIVAL CREVICE TO *BACTEROIDES GINGIVALIS* AND *BACTEROIDES MELANINOGENICUS* SS. *INTERMEDIUS* BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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It is now well established that anaerobic bacteria, especially the black pigmented *Bacteroides* species are involved in the aetiology of chronic periodontal disease (1). *Bacteroides gingivalis* (formerly oral *B. asaccharolyticus*) is one of the predominant species isolated from advanced periodontal disease (2) and recent evidence suggests it may be playing an important pathogenic role (3, 4). As well as the enzymes and toxic metabolites that such bacteria produce, immunopathologic mechanisms which are stimulated by bacterial antigens may be important in the disease process (5).

Recent reports have described the measurement of serum antibodies to *B. gingivalis* (6, 7) and Kagan (3) has assessed local immunity by demonstrating the specificity of plasma cells in the gingival tissue to *B. gingivalis* by immunofluorescence technique. These results suggest that it might be possible to assess the activity of periodontal disease by measuring the immune response to potentially pathogenic organisms.

The study reported here explores the possibility that the detection and quantitation of antibodies produced locally in the ginigival crevice against *B. gingivalis* and *B. melaninogenicus* ss. *intermedius* another species implicated in periodontal disease, might give an indication of the disease activity. Bacteroides organisms have been shown to possess species specific antigens in their outer membranes and these can be detected quantitatively by an enzyme-linked immunosorbent assay (ELISA) (8).

Methods: Eighty two patients presenting with varying degrees of gingivitis and periodontotis, were included in the survey. Details of age, sex, recent or current antimicrobial therapy and other treatment were noted, but the major clinical criteria were measurement of Pocket Depth and Bleeding Index by the criteria of the WHO Technical Report (9). These measurements were performed after sample collection.

Samples were collected by washing the gingival crevice using a technique similar to that described by Skapski and Lehner (10). The site was isolated and dried. A microsyringe (SGE Ltd., Melbourne, Australia, Model 50A, 50 μl capacity) fitted with a dome tipped needle was used to introduce 15 μl of buffer (50 mM Na phosphate buffer pH 7.4 containing 0.15 M NaCl and 0.05% Tween 20: PBST) into the gingival crevice. Washings were withdrawn and more buffer added until 100 μl of fluid had been collected. The sample was centrifuged for 1.5 min at 9500 *g* in a Beckman Microfuge B and the pellet was discarded. The antibody-containing supernate was stored at -20 °C.

The enzyme linked immunosorbent assay (ELISA) was similar to that described earlier for assay of rabbit immunoglobulins (8). Outer membrane complex antigens were prepared from *B. gingivalis* ATCC 33227 and *B. melaninogenicus* ss. *intermedius* NCTC 9338 by the EDTA, 45 °C, sonic bath treatment and used without dialysis at a concentration of 30 μg/ml to coat wells (50 μl) in a microtitre plate as described previously (8). Dilutions (1 in 10, 20, 40 and 80) of crevice washing in PBST were added to the wells and incubated for 4 h at room temperature. After washing, anti-human IgG, IgM and IgA alkaline phosphatase conjugates (Miles, Slough, UK) were added and incubated for 16 h at room temperature. The dilutions of Ig conjugates used were the highest dilution that gave an A_{405} reading (Titertek Multiskan, Organon Teknika, St Neots, Camb., UK) of 1.4 when titrated against IgG, M and A myeloma sera diluted to 200 μg/ml (supplied by Blood Transfusion Service, Royal Infirmary, Edinburgh) by our standard ELISA method (8). Results were read in a multiskan and expressed as matrix values of 0–9 where one matrix unit corresponded to a range of 0.2 absorbance units at 405 nm.

Results and discussion: In preliminary experiments the background level in the ELISA was investigated: dilutions of crevicular washings which gave apparently high levels of IgG to *B. gingivalis* or *B. melaninogenicus* ss. *intermedius* were reacted aginst antigens prepared from the non oral *B. fragilis* (NCTC 9344). An A_{405} reading of less than 0.2 (matrix value 0) was found if a dilution of 1 in 10 was made of the gingival washing. In subsequent assays a reading of greater than 0.2 (matrix value 1 or more) for a 1 in 10 dilution of crevicular fluid was judged as a significant level of Ig.

In the 109 samples taken from 82 patients no IgA could be detected and IgM was present in trace amounts in only a few patients (ELISA reading approaching A_{405} of 0.2). Significant levels of IgG were detected in 103 (95%) of samples. Specific IgG for *B. gingivalis* was found in 95 samples (87% of total) and for *B. melaninogenicus* ss. *intermedius* in 82 (75% of total).

The Ig levels ranged between matrix values of 1–9. Most fell in the lower end of the range (values of 1–4) but 30 samples had a level of IgG which gave a matrix value of 5 or greater. These high levels of IgG to *B. gingivalis* were found in 11 samples (10%) and to *B. melaninogenicus* ss. *intermedius* 19 samples (17%). In the samples which had a high level of antibody to one species there was a correspondingly low level of antibodies to other species. Of the 30 samples which gave an ELISA level of 5 or greater for one antibody all but 2 of these had a level at least two-fold that of the other antibody. Nine samples had four-fold or greater variation between the antibody levels. This suggests that it is uncommon to have high antibody levels against both of these *Bacteroides* species in the same individual. There was no correlation between IgG

level and the clinical parameters of pocket depth and bleeding index. Within the same mouth there were local differences between the sites sampled. Examples of five patients showing this variation are summarised in the table. These results confirm that the technique is measuring antibodies in a local exudate rather than simply a serum exudate.

Local variation in IgG levels from two or more sites within the same mouth

Patient	Sample site[a]	Pocket depth (mm)	Bleeding index[b]	IgG level[c] to: *B. gingivalis*	IgG level[c] to: *B. melaninogenicus* ss. *intermedius*
1	Upper right 7	5	1	8	4
	Upper left 7	4	1	5	2
	Upper right 2	2	0	0	1
2	Upper right 3	3	0	0	3
	Upper left 2	2	1	0	1
3	Upper right 3	4.5	1	1	4
	Upper right 2	5	1	1	2
4	Upper right 4	4	1	1	2
	Lower right 5	10	1	1	4
5	Upper right 5	7	1	9+	1
	Upper right 2	5	1	3	1

a: Counting from the centre. b: 1 = bled on gentle probing; 0 = did not bleed. c: IgG level expressed as a matrix value of the A_{405} reading of the 1 in 10 dilution of crevicular washing in the standard ELISA.

In this pilot study, most patients had samples taken on only one occasion. One patient from whom samples were taken more than once may be worthy of note. A patient (female, 23 years) presented with juvenile periodontitis in 1974 and has received periodontal treatment extensively since that time. Two samples taken at intervals of 12 weeks had ELISA levels of antibodies to *B. gingivalis* and *B. melaninogenicus* ss. *intermedius* of 1 and 9 respectively for the first sample and 1 and 7 for the second sample, indicating high levels of *B. melaninogenicus* ss. *intermedius* antibodies present.

Although these results are of a preliminary nature, there appears to be an indication that the measurement of the local antibody response to bacteria which may be involved in the aetiology of periodontal disease might give a quantitative and reproducible index of the activity of the organisms in the disease process. So far, an investigation has been made into the levels of only two antibodies. Many other species of bacteria have been implicated in the aetiology of periodontal disease and antibody levels to these should be assayed. Recently a report has been published showing increased levels of serum antibodies to *Treponema denticola*, an anaerobic spirochaete, in cases of advanced periodontitis (11).

We suggest that this assay for specific local antibodies secreted into the gingival crevice/periodontal pocket should be extended to a controlled study and results might shed more light onto the complex pathologic mechanisms that exist in periodontal disease.

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Immunological Analysis of the EDTA-soluble Antigens of *Clostridium difficile* and Related Species

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Antigens were extracted with EDTA from 32 strains representing 10 species of *Clostridium*. When these antigens were examined by an enzyme-linked immunosorbent assay, marked cross-reactions were observed between *C. difficile*, *C. sordellii* and *C. bifermentans*. The cross-reactive antigen, visualized by crossed immunoelectrophoresis, was carbohydrate.

INTRODUCTION

Until recently *Clostridium difficile* was regarded as an uncommon, non-pathogenic organism. It is part of the normal faecal flora of many neonates but is rarely found in adults (Larson *et al.*, 1978). Since 1977, however, there has been an increasing number of reports to suggest that this organism is the major, if not the only, cause of antibiotic-associated colitis (Larson, 1979; Bartlett, 1979). There have also been recent reports that *C. difficile* is involved in chronic inflammatory bowel disease (Lamont & Trnka, 1980; Bolton *et al.*, 1980).

Two methods are currently available for the detection of *C. difficile* in the faeces: (i) it can be cultured on a highly selective medium (George *et al.*, 1979); (ii) its toxin can be demonstrated in a faecal filtrate by a cytopathic effect on tissue monolayers. This effect can be neutralized with *C. sordellii* antitoxin (Rifkin *et al.*, 1977). There is a need to develop an immunological method for the identification of *C. difficile* in faecal smears by direct microscopy. Before such a technique can be developed, cross-reactions between serologically related species must be investigated. Furthermore, a knowledge of the antigens of this species might allow the development of a serotyping scheme to elucidate the epidemiology of *C. difficile* (see Larson, 1979).

The present study examines serological relationships of *C. difficile*, *C. sordellii* and *C. bifermentans* by enzyme-linked immunosorbent assays and crossed immunoelectrophoresis. *Clostridium sordellii* and *C. bifermentans*, regarded by many as the same species, are commonly encountered in soil, sewage and the large bowel of man and animals. They are uncommon pathogens, but are occasionally associated with gas gangrene (Willis, 1969, 1977).

METHODS

Organisms and growth conditions. The strains of bacteria used were: *C. difficile*, N1, N3, N4, N5 and N6 (obtained from Dr S. Hafiz, University of Sheffield Medical School), MPRL2, 15, 40, 50 and 59 (isolated in the Bacteriology Department, University of Edinburgh), RIE11831 (isolated in the Royal Infirmary, Edinburgh), FAL11456 (isolated at Fife Area Laboratories, Kirkcaldy) and NCTC 11223; *C. sordellii*, NCTC 1340, NCTC 2914, NCTC 6800, NCTC 6801, NCTC 6927, NCTC 6929 and NCTC 8780; *C. bifermentans*, NCTC 1341, NCTC 6928 and three laboratory strains; *C. perfringens*, NCTC 1869, NCTC 3110, NCTC 3180, NCTC 8237, NCTC 8239 and one laboratory strain; *C. paraperfringens*, a laboratory strain; *C. fallax*, NCTC 8380; *C. sporogenes*, NCTC 8594; *C. novyi*, a laboratory strain; *C. chauvoei*, NCTC 8070; *C. absonum*, a laboratory strain.

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All strains were cultured from a stationary phase broth culture in modified Robertson's cooked meat medium (Watt, 1973). Proteose peptone/yeast extract broth (Holbrook *et al.*, 1977) was inoculated (2%, v/v) and cultured for 16 h at 37 °C by the standard anaerobic procedure of Collee *et al.* (1972). Sporulation was negligible under these conditions.

Extraction of antigens. Bacteria, cultured in 100 ml medium (see above), were harvested by centrifugation at 2400 *g* for 20 min at room temperature and washed twice in phosphate buffered saline (PBS, 50 mM-sodium phosphate buffer, pH 7·4 containing 0·15 M-NaCl). The pellet was suspended in 4 ml PBS containing 10 mM-EDTA and incubated for 30 min at 45 °C. The extracted cells were removed by centrifugation (10000 *g*, 10 min, 4 °C) and the supernatant fluid was dialysed twice against 2 l volumes of PBS over 20 h at 4 °C. Protein concentrations, after dialysis, were measured by the Lowry method. Antigens were stored at −20 °C.

Preparation of antisera. Antisera against u.v.-killed, whole, washed bacteria were raised in New Zealand White rabbits as described previously for *Bacteroides* spp. (Poxton, 1979). *Clostridium difficile* NCTC 11223 and *C. sordellii* NCTC 1340 were used at the standard doses of 10^9 cells throughout the injection schedule. However, for the toxigenic *C. sordellii* NCTC 8780, the first three doses were 10^6 cells, increasing to three doses of 10^7 cells in the second week and boosted by one dose of 10^7 cells in the fourth week.

Absorption of sera. Cells for absorption were cultured on blood agar for 18 h at 37 °C. They were carefully scraped from the surface, suspended in PBS and washed twice in PBS. Undiluted serum was added directly to the washed pellet at a ratio of 1 ml of serum to bacteria from ten plates. The mixture was gently homogenized and incubated at 37 °C for 30 min. Bacteria were removed by centrifugation at 2400 *g* for 20 min at 20 °C.

Micro-enzyme-linked immunosorbent assay (ELISA). The EDTA-extracted antigen was diluted to a concentration of 30 μg protein ml^{-1} in antigen buffer just prior to coating wells of microtitre plates. The method was as described previously (Poxton, 1979) except that the results were read in a Titretek Multiskan (Organon Teknika, St Neots). The end-point of the titration of antiserum (titre) was the first dilution with an A_{405} value of less than 1·6.

Crossed immunoelectrophoresis (CIE). The procedure was essentially the same as that described by Weeke (1973*a*). The EDTA-extracted antigens were made up to 2 mg protein ml^{-1} in PBS and equilibrated by dialysis in CIE sample buffer (see below). Triton X-100 was added to the dialysed antigen at a concentration of 1% (v/v). Antigens (12 μl) were examined by CIE on 5 × 5 cm glass slides using barbital/glycine/Tris buffer pH 8·8 (Weeke, 1973*b*). Buffer was used undiluted (ionic strength 0·08) in the electrode reservoirs, but in the gels and sample buffer it was diluted 1 in 4 with distilled water, and Triton X-100 was added to a concentration of 1% (v/v). Gels contained agarose (BDH electrophoresis grade) at a concentration of 1% (w/v). Electrophoresis was at 12·5 V cm^{-1} for 1·5 to 2 h in the first dimension and at 12 V cm^{-1} for 16 h in the second dimension, both at 4 °C. Gels were pressed, washed and stained with Coomassie blue as described by Weeke (1973*b*), or stained with the periodate–Schiff's reagents (Segrest & Jackson, 1977).

Treatment of antigens. The following treatments were performed on the antigens and the effects on the CIE profiles were examined: (i) heat, 121 °C for 15 min; (ii) formalin, 20% (w/v) formaldehyde for 16 h at 20 °C followed by dialysis against PBS for 18 h at 4 °C; (iii) periodate, 0·1 M-$NaIO_4$ for 16 h at 20 °C in the dark; excess periodate was consumed by the addition of ethylene glycol and this was followed by dialysis against PBS for 18 h at 4 °C. Untreated controls were included for treatments (ii) and (iii).

RESULTS

EDTA extracts were prepared from 32 strains representing ten species of *Clostridium*. Antisera raised against whole, washed cells of *C. difficile* NCTC 11223, *C. sordellii* NCTC 1340 and *C. sordellii* NCTC 8780 (a toxigenic strain) were titrated with the extracts in an enzyme-linked immunosorbent assay (ELISA). Cross-reactions were observed only between *C. difficile*, *C. sordellii* and *C. bifermentans* strains. The antigens extracted from these three species gave titres ranging between 3200 and 25600 when reacted with the antisera raised against the *C. difficile* strain and the two *C. sordellii* strains. A titre of less than 100 was obtained when antigens prepared from *C. perfringens* Types A to E, *C. paraperfringens*, *C. fallax*, *C. sporogenes*, *C. novyi*, *C. chauvoei* and *C. absonum* were reacted with *C. difficile* or *C. sordellii* antisera. It was impossible to differentiate beween *C. difficile*, *C. sordellii* and *C. bifermentans* when EDTA-extracted antigens were titrated with unabsorbed sera. In an attempt to reduce the cross-reactions between species the sera were absorbed with whole cells of the cross-reactive species. This resulted in a more specific reaction but it was accompanied by a marked reduction in the titre of homologous

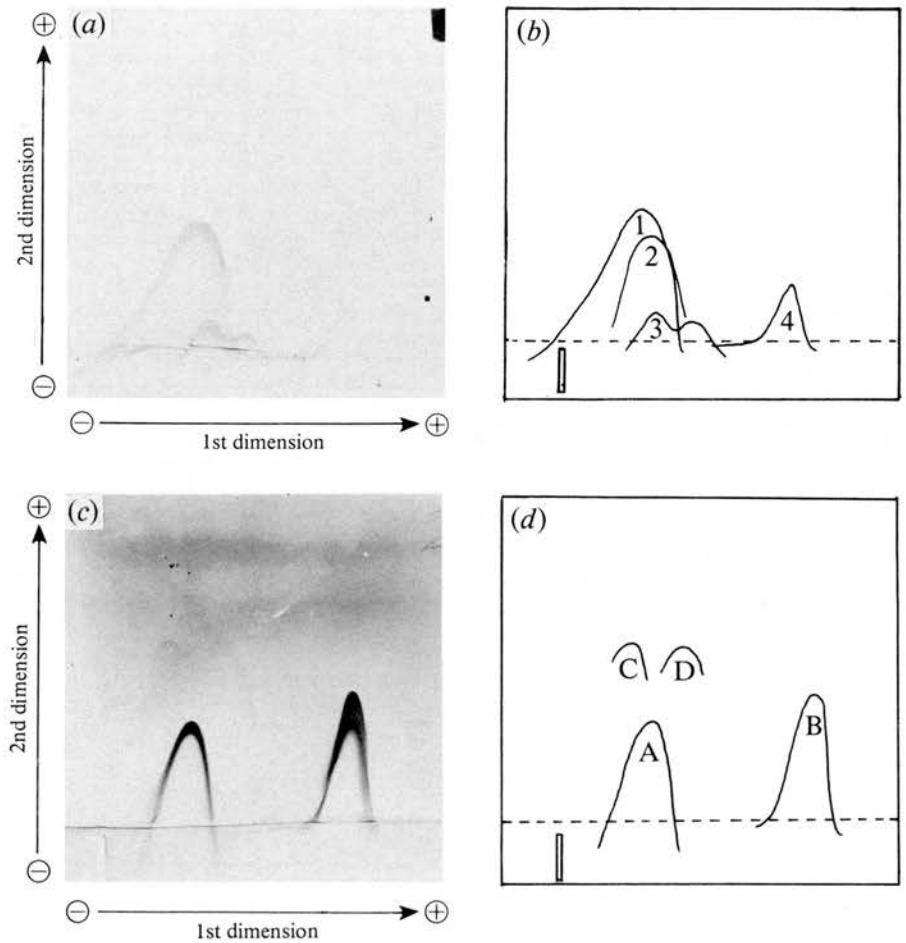

Fig. 1. Crossed immunoelectrophoresis of EDTA-extracted antigens from *C. difficile* NCTC 11223 and *C. sordellii* NCTC 8780 against homologous whole cell antisera: (*a*, and drawing *b*) 12 µl antigen from *C. difficile*, after electrophoresis in the 1st dimension, was run into agarose containing 63 µl antiserum; (*c*, and drawing *d*) 12 µl antigen from *C. sordellii* was run into agarose containing 500 µl antiserum.

antigen/antibody reaction. This suggested that there were some species-specific antigens in the EDTA extracts but that other antigens were shared between these three species.

The antigen/antibody reactions demonstrated by ELISA were visualized by crossed immunoelectrophoresis (CIE). Figure 1 shows that the homologous antigen/antibody reactions for *C. difficile* NCTC 11223 and for *C. sordellii* NCTC 8780 are quite different from one another. *Clostridium sordellii* NCTC 1340 gave a reaction that was almost identical with that of strain NCTC 8780. In similar tests with *C. bifermentans* antigens run against *C. sordellii* antisera, the patterns were indistinguishable from those obtained with *C. sordellii* antigens.

Table 1 indicates the number and position of precipitin lines obtained with antigens prepared from 13 strains of *C. difficile* run against *C. difficile* NCTC 11223 antiserum. The positions of these precipitin lines are given by reference to the lines shown in Fig. 1 (*b*). Line 2 is common for all strains tested, line 4 occurs frequently and lines 1 and 3 are less frequently demonstrable.

Clostridium difficile antigens reacted with *C. sordellii* antisera to give line 2 on all

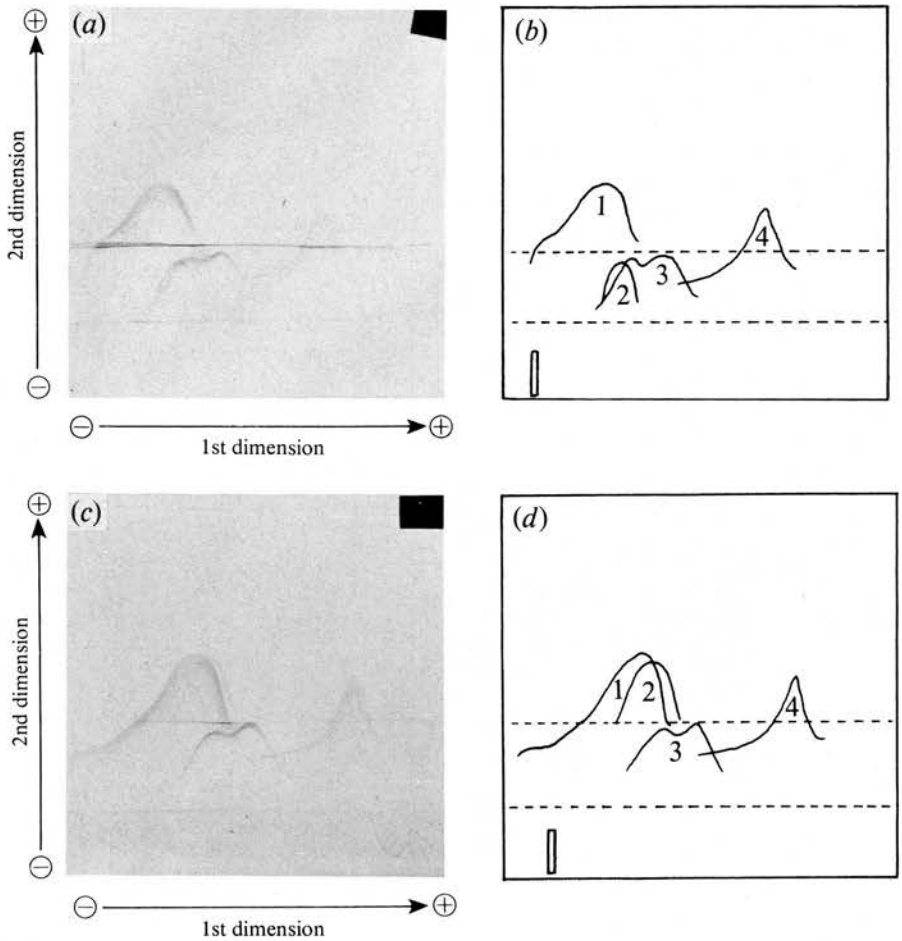

Fig. 2. Intermediate-gel crossed immunoelectrophoresis of *C. difficile* NCTC 11223 EDTA-extracted antigens: (*a*, and drawing *b*) 12 μl antigen, after electrophoresis in 1st dimension, was run through a 1 cm intermediate gel containing 63 μl antiserum to *C. sordellii* NCTC 8780 into a gel containing 48 μl *C. difficile* NCTC 11223 antiserum; (*c*, and drawing *d*) as above except the intermediate gel did not contain any serum. Peaks are numbered as in Fig. 1 (*b*).

Table 1. *Variation in EDTA-extracted antigens from 13 strains of C. difficile run against NCTC 11223 antiserum in crossed immunoelectrophoresis*

Strain of *C. difficile*	Precipitin lines in EDTA extract*
NCTC 11223	1 2 3 4
N1	1 2 4
N3	1 2
N4	2 4
N5	2 4
N6	2 3
RIE11831	2 4
FAL11456	1 2 4
MPRL2	2 3 4
MPRL15	2 4
MPRL40	1 2 4
MPRL50	2 4
MPRL59	2 4

* Precipitin lines numbered as in Fig. 1 (*b*).

occasions. No other precipitin line occurred in these tests. However, *C. sordellii* antigens reacted with the *C. difficile* antiserum to give two lines in positions A and B shown in Fig. 1 (*d*). This suggested that line 2 and line A are lines of cross-reaction and may be identical. This was further investigated by exploiting the intermediate gel procedure of Axelsen (1973).

Clostridium difficile antigen was run through an intermediate gel containing *C. sordellii* antiserum and then into a gel containing *C. difficile* antiserum (Fig. 2*a*, *b*). In a control, the intermediate gel contained no antiserum (Fig. 2*c*, *d*). Lines 1, 2, 3 and 4 developed in the test and control. However, the presence of the *C. sordellii* antiserum in the intermediate gel held back the precipitin line 2. The other lines were identical in the test and the control. The position of line 3 at the intermediate gel level is explained by back diffusion of the antiserum.

The converse experiment was done with *C. sordellii* antigen run through an intermediate gel containing *C. difficile* antiserum into gel containing *C. sordellii* antiserum. In this case both lines A and B (see Fig. 1*d*) were held back by the serum in the intermediate gel.

The EDTA-extracted antigen of *C. difficile* used in these studies contained approximately 90% protein and 10% carbohydrate as estimated by the Lowry method and by the method of Dubois *et al.* (1956), respectively. Examination by sodium dodecyl sulphate–polyacrylamide gel electrophoresis showed that it contained at least 25 polypeptides. In an attempt to characterize the antigens that were visualized by CIE a number of chemical and physical treatments were performed (see Methods for details). (i) After heating, only line 2 remained. (ii) After treatment with formalin, only line 2 was unchanged; lines 3 and 4 were lost and line 1 was diffuse and changed in shape. (iii) After treatment with periodate, lines 2 and 4 were lost. In an untreated control, which was dialysed in the same way as in treatments (ii) and (iii), line 4 was also lost. This suggested that the antigen which precipitated in line 4 was either extremely labile or was lost during extensive dialysis. All lines except line 4 stained with the periodate–Schiff's reagents in an untreated control that had not been dialysed.

DISCUSSION

If the composition of the clostridial cell surface is similar to that of other Gram-positive bacteria, the surface antigens will include a range of proteins and possibly capsular material, and teichoic acid components or their analogues associated with the cell wall and cytoplasmic membrane. In the present study, EDTA was chosen to solubilize antigens from *C. difficile* and related species because it should solubilize protein and perhaps membrane teichoic acid without the breakage of any covalent bonds and without the extraction of wall teichoic acids; a considerable advantage is that autolysis is apparently inhibited by EDTA (unpublished observation).

All of the strains of *C. difficile, C. sordellii* and *C. bifermentans* examined share a common EDTA-extractable antigen which is probably carbohydrate; it is thermostable, inactivated by periodate and stains with the periodate–Schiff's reagent. In addition, all the strains of *C. difficile* had at least one other protein or possibly glycoprotein antigen complex that appears to be specific for *C. difficile.* It should be possible to exploit this finding to raise a specific *C. difficile* antiserum that will not cross-react with the *C. sordellii–bifermentans* group. In addition, the EDTA-extractable antigens may provide a basis for a sero-typing system of epidemiological value in tracing *C. difficile* infections.

The known serological relationships between *C. bifermentans* and *C. sordellii* (Huang, 1959, cited by Willis, 1977) are reflected in our finding that the EDTA-extracted antigens of these two species were indistinguishable by ELISA and CIE procedures. The relationship of *C. difficile* to the *bifermentans–sordellii* complex, suggested by the cross-neutralization of its toxin by *C. sordellii* antitoxin, is therefore paralleled by the somatic antigenic relationships that our studies have revealed.

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DEMONSTRATION OF SHARED ANTIGENS IN THE GENUS *CLOSTRIDIUM* BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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SUMMARY. Antigens prepared from several strains of each of 10 *Clostridium* species were used in an indirect enzyme-linked immunosorbent assay with antisera raised against whole cells of a representative strain from each of the 10 species killed by ultra-violet irradiation. With the exception of *C. cadaveris*, the antisera gave similar results with antigens prepared from all strains of the homologous species. Antigens prepared from 13 other clostridial species were then investigated in an ELISA system with the 10 representative antisera. The results showed many cross-reactions, particularly in the *C. perfringens* group, the *C. difficile/sordelli* group and the *C. botulinum/novyi/sporogenes* group.

INTRODUCTION

Bacteria in the genus *Clostridium* are subdivided into species according to a range of morphological and biochemical criteria that include shape, position of spore, the ability to digest proteins and ferment various sugars and the production of a range of extracellular enzymes and toxins. Several identification schemes are available, e.g. those of Holdeman, Cato and Moore (1977) and Willis (1969), but in practice a number of isolates prove difficult to identify. Many species are closely related, and morphological and biochemical variations occur within strains of the same species and even within a subculture of the same strain. Toxigenic and non-toxigenic variants of the same species occur and some species can be distinguished only by differences in the toxins they produce (Cruickshank *et al.*, 1975).

In human and verterinary medicine, immunological methods have been exploited to detect and identify clostridia. Immunofluorescent antibody (IFA) kits are commercially available for *C. botulinum* types A, C and E, *C. septicum*, *C. chauvoei*, *C. novyi* and *C. tetani* (Wellcome Diagnostics, Dartford, England), for the detection of *C. botulinum* types A, B and F in suspected infant botulism (Hatheway and McCroskey, 1981) and for *C. difficile* in cases of antibiotic associated pseudomembranous colitis (Hubert, Ionesco and Sebald, 1981). Several studies of antigenic relationships in the genus *Clostridium* have been based on agglutination tests (Mandia, 1955) or empirical immunofluorescence reactions (Walker and Batty, 1964; Batty and Walker, 1965; Lynt, Solomon and Kautter, 1971) with a range of antisera raised against heat-killed or

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formalin-killed organisms. More recently, we presented evidence for antigenic relationships between *C. difficile* and *C. bifermentans* and *C. sordelli* (Poxton and Byrne, 1981*a*) based on surface antigens extracted by EDTA. These were demonstrated by an enzyme-linked immunosorbent assay (ELISA) and crossed immunoelectrophoresis and we were able to provide guidelines for the detection of *C. difficile* by immunotechniques (Poxton and Byrne, 1981*b*).

The work reported here extends these studies to the majority of medically important species of *Clostridium*.

Materials and methods

Bacterial strains. The following strains were obtained from the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale Avenue, London NW9 5HT: *Clostridium bifermentans* NCTC1341; *C. butyricum* NCTC7423; *C. chauvoei* NCTC8070; *C. difficile* NCTC11223; *C. fallax* NCTC8380; *C. histolyticum* NCTC nos. 503, 7123 and 7124; *C. novyi* type A NCTC538, type C NCTC9747 and type D NCTC8350; *C. perfringens* type A NCTC8237, type B NCTC3110, type C NCTC3180 and type E NCTC8084; *C. septicum* NCTC547, *C. sordelli* NCTC8780; *C. sporogenes* NCTC8594; *C. tertium* NCTC541 and *C. tetani* NCTC249. *C. irregularis* NCIB11830 (ATCC25756) was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. The strains of *C. botulinum* were from the collection in the Botulinum Laboratory, Unilever Research, Sharnbrook, Bedford: type A, strain nos. 1, 7, 8, 9; type B (proteolytic) strain nos. 25, 26, 27; type B (non-proteolytic) strain nos. 188, 194, 229; type C, strain no. 205; type D, strain no. 154; and type E, strain nos. 34, 118, 126, 250. Other strains included our own laboratory cultures of *C. cadaveris* MPRL34, MPRL506, 50233; *C. irregularis* 4403; *C. nexile* MPRL39; *C. novyi* type A GR2A, GR3A; *C. novyi* type B GR2B; *C. paraputrificum* CP3; *C. perenne* MPRL95; *C. sporogenes* 24, 33; *C. subterminale* MPRL303; and *C. tertium* CT1, CT2. Others were obtained from the following sources: *C. absonum* Ha 9103 and *C. paraperfringens* 2227 from Professor S. Nishida, Kanasawa University, Japan and *C. perfringens* type D, L85 from the late Professor C. L. Oakley, University of Leeds. All strains were fully authenticated by performing the biochemical tests described in the Virginia Polytechnic Institute Anaerobe Laboratory Manual (Holdeman *et al.*, 1977).

Culture of strains and preparation of EDTA extracts. Lyophilised cultures were inoculated into Robertson's Cooked Meat Broth (10 ml) and incubated anaerobically with CO_2 10% for 16 h at 37°C. These cultures (0·2 ml volumes) were used to inoculate 100-ml volumes of proteose peptone-yeast extract broth (Holbrook, Duerden and Deacon, 1977) which were incubated anaerobically with CO_2 10% for 16 h at 37°C. Spore formation, as determined by phase contrast microscopy, was negligible under these conditions. Antigens were prepared from the 100-ml cultures by extraction with 10mM EDTA by the method described previously (Poxton and Byrne, 1981*a*) except that the dialysis step was found to be unnecessary and was omitted.

Preparation of antisera. Antisera were raised in New Zealand White rabbits by intravenous injection of whole, washed, killed bacteria during a 5-week period (Poxton, 1979). The bacteria were killed by ultraviolet irradiation and 10^9 organisms were used for each injection except for the toxigenic strain of *C. sordelli*, NCTC8780 (10^6–10^7 organisms, see Poxton and Byrne, 1981*a*).

Indirect enzyme-linked immunosorbent assay (ELISA). The EDTA extracts were diluted to contain protein 30 μg/ml in antigen buffer and used to coat wells in flat-bottomed microtitre plates. The ELISA technique was performed as described earlier (Poxton, Brown and Collee, 1982).

Reading and presentation of results. In all titrations, a homologous reaction was included and heterologous reactions were compared directly with this. A reaction was considered significant if the titre was within six doubling dilutions (T/32) of the titre of the reference reaction obtained between a reference antiserum and the homologous antigen preparation. The results are expressed as a fraction of the homologous reference titre. The size of the symbol in the figure is proportional to the degree of the reaction or cross-reaction, on this basis. A reaction at a titre of less than 1/32 of the homologous titre was considered insignificant.

RESULTS

Reference homologous reactions

Initially, EDTA extracts prepared from 10 species of *Clostridium* were used as antigens in an indirect ELISA system with their homologous antisera. During the course of the study, these homologous reactions were included as controls whenever a specific antiserum was used and the range of titres (table) reflects the variation found in repeated titrations.

TABLE

Reactions of EDTA antigens with homologous antisera in an indirect ELISA system

Reference antiserum raised against (species and strain no.)	Range of homologous titres (T)	Number of strains reacting to a titre of							Total number of strains tested
		T	T/2	T/4	T/8	T/16	T/32	<T/32	
C. cadaveris (MPRL34)	12800–25600	1					1	1	3
C. chauvoei (NCTC8070)	12800–25600	1	1						2
C. difficile (NCTC11223)	25600–51200	6	25	3					34
C. histolyticum (NCTC503)	6400–51200	2	1						3
C. novyi, type A (NCTC538)	25600–102400	1				1	1		3
C. perfringens, type A (NCTC8237)	12800–51200	2	4	1					7
C. septicum (NCTC547)	12800–25600	3	2						5
C. sordelli (NCTC8780)	12800–25600	8							8
C. sporogenes (NCTC8594)	6400–25600	1		2					3
C. tertium (NCTC541)	1600–3200	1	2						3

Tests with representative strains

Antigens were prepared from 61 other strains of *Clostridium* belonging to the 10 species listed in the table; they were then used to compare the titres with these antigens and the 10 antisera (now referred to as reference antisera) with those of the homologous reactions. The results indicated that most of the antigens gave titres within one or two doubling dilutions of the homologous reaction (table). Only the antiserum against *C. cadaveris* failed to react significantly with either of the other two strains tested. The reactions of the *C. novyi* type A strains were not as strong as those of the other species but a reaction was considered significant if the titre was within six doubling dilutions (T/32) of that of the homologous reaction (see *Methods*).

Studies of cross-reactions

With the knowledge that nine of the reference antisera reacted strongly with antigens prepared from several strains of the same species, all 10 reference antisera were used to investigate cross-reactions with antigens from at least two strains of 13 other clostridial species, including five different types of *C. perfringens* and *C. botulinum*, and four of *C. novyi*. The results are summarised in the figure. They are expressed as a fraction of the titre obtained with the reference antiserum and the homologous antigen (see *Methods*). Three areas of major cross-reactions were identified: in the *C. perfringens* group, in the *C. difficile/sordelli* group, and in the *C. botulinum/novyi/sporogenes* complex.

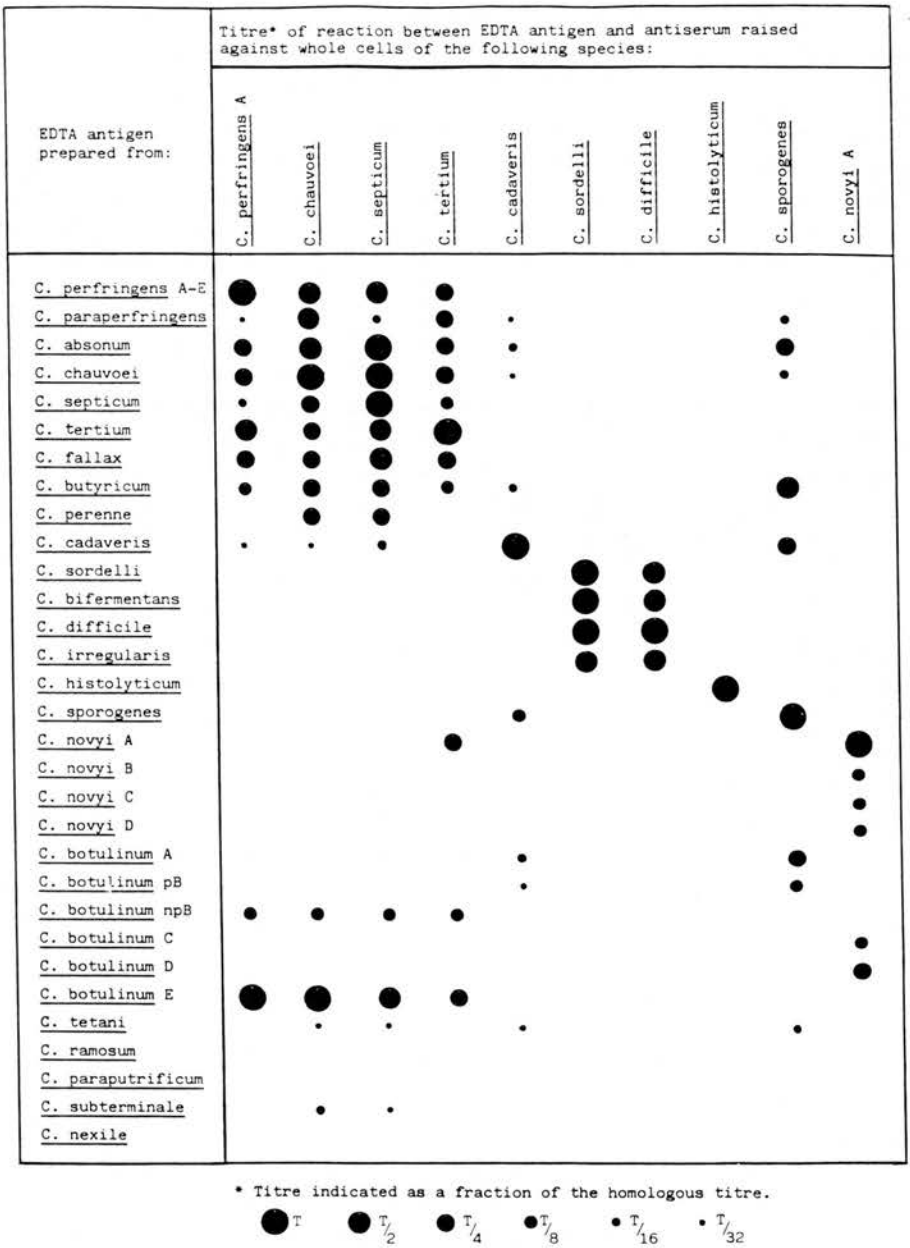

FIG.—Cross-reactions within the genus *Clostridium* in an indirect ELISA test with EDTA-extracted antigens and whole cell antisera.

DISCUSSION

The *C. perfringens* group included the first 10 species listed in column 1 of the figure. From previous work, some of the cross-reactions might have been expected, although others were not. All types of *C. perfringens* (A–E) were indistinguishable by

this test and they reacted strongly with *C. chauvoei*, *C. septicum* and *C. tertium* antisera as well as with the *C. perfringens* type A reference antiserum. *C. perfringens*, *C. paraperfringens* and *C. absonum* are related biochemically and antigenic relationships could be expected. *C. chauvoei* and *C. septicum* are also inter-related but it was unexpected to find that these species reacted strongly with the reference antisera against *C. perfringens* and *C. tertium*. The strong reactions of the above species, together with those of *C. fallax* and *C. butyricum* with the first four reference antisera, and to a lesser extent with the *C. cadaveris* and *C. sporogenes* antisera, confirmed the cross-reactions. Antigens prepared from *C. botulinum* type B (non-proteolytic) and type E were also included in this group.

The *C. sordelli/bifermentans* and *C. difficile* group were studied in detail before (Poxton and Byrne, 1981*a*) but in this extended survey we included the species *C. irregularis*. We have been able to obtain only two strains of *C. irregularis*, one isolated in our own laboratory, strain 4403, and one from the NCIB (strain no. 11830, which is the same as ATCC25756). Both strains were indistinguishable from *C. difficile*, *C. sordelli* and *C. bifermentans* in our ELISA system and shared the same major carbohydrate antigens as these strains (I. R. Poxton, unpublished results). The antigens from these four species did not cross-react with any other antisera, nor did the *C. sordelli* or *C. difficile* antisera cross-react with any other antigen.

It is well established that organisms in the *C. botulinum/novyi/sporogenes* complex possess some biochemical and cultural similarities (Smith, 1977). *C. botulinum* type A and type B (proteolytic) are biochemically and culturally indistinguishable from *C. sporogenes*; only the toxins separate them. This relationship was confirmed by the ELISA technique which showed the presence of shared antigens. Three of four type-A strains and all of three proteolytic type-B strains reacted with the *C. sporogenes* reference antiserum. Similarly, *C. botulinum* types C and D are indistinguishable from *C. novyi* type A (except by toxin), and this was borne out in this study. The unexpected result in this group was the demonstration of cross-reactions between *C. botulinum* type B (non-proteolytic) and type E antigens with the first four reference antisera. Taken in isolation this result may be questioned; however, non-proteolytic type B and type E *C. botulinum* are distinguished only by toxin production (Smith, 1977) and all of three strains of non-proteolytic type B and all of four strains of *C. botulinum* type E gave identical results with the four reference antisera, indicating that the result was consistent.

The antisera prepared from *C. sporogenes* and *C. cadaveris* antigens had remarkably similar patterns of specificity. The *C. cadaveris* antiserum, however, may be atypical as antigens prepared from the other two strains did not react significantly with it.

This study has demonstrated some unexpected shared antigens in the genus *Clostridium* but has confirmed others. The EDTA preparation used as antigen for the *C. difficile/sordelli* group contained proteins and carbohydrate extracted from the cell surface (Poxton and Byrne, 1981*a*; Poxton and Cartmill, 1982); the previously unrecognised cross-reactions demonstrated in this study were presumably due to the presence of heat- and formaldehyde-labile antigens not detected in earlier studies. It is difficult to compare our results with those obtained in previous studies which have been used to separate clostridia by immunofluorescent antibody techniques, for such tests to be satisfactory they must be specific, and cross-reacting antibodies were removed by

absorption. The assay system we have used is relatively non-selective and highly sensitive. We suggest that the results presented here may provide useful guidelines when designing immunological methods for the detection of clostridia and may also help to define taxonomic relationships in the genus. A detailed investigation at the molecular level of some of the more important areas of cross reactions is currently being made.

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Demonstration of the Common Antigens of *Clostridium botulinum*, *C. sporogenes* and *C. novyi* by an Enzyme-linked Immunosorbent Assay and Electroblot Transfer

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EDTA extracts were prepared from whole cells of 16 strains of *Clostridium botulinum* (types A–E), 6 strains of *C. novyi* (types A–D) and 3 strains of *C. sporogenes*. They were reacted in an enzyme-linked immunosorbent assay (ELISA) with antisera raised against whole, UV-killed cells of *C. sporogenes* and *C. novyi* type A. Results showed significant cross-reactions between *C. sporogenes* antiserum and the *C. botulinum* type A (three out of four strains), proteolytic type B (all strains) and one type E strain, and between *C. novyi* type A antiserum and *C. botulinum* types C and D. All the *C. sporogenes* and *C. novyi* strains reacted with their homologous antiserum; these two species showed no cross-reactions. All the reactions were investigated further by running the EDTA extracts on SDS-polyacrylamide gels. The separated molecules were electrophoretically transferred to nitrocellulose membranes, reacted with antiserum and complexes visualized with horseradish peroxidase conjugate reagents. Only those extracts that reacted significantly in the ELISA gave a pattern of cross-reactive antigen bands and the number of bands and intensity of stain closely paralleled the strength of the ELISA reaction.

INTRODUCTION

The heterogeneous group of clostridia collectively known as *Clostridium botulinum* are alike in that they all produce toxins with a similar pharmacological action. In taxonomic terms, however, strains of *C. botulinum* can be divided into four reasonably distinct groups: Group I, proteolytic strains with serologically distinct A and B toxins; Group II, non-proteolytic strains producing toxins of types B, E and F; Group III, strains producing toxins of types C and D; and Group IV, proteolytic, non-saccharolytic, toxigenic type G strains (Smith, 1977). In culture and biochemistry, excluding toxin production, Group I strains are indistinguishable from *C. sporogenes*; accordingly *C. sporogenes* is considered by some workers to be a non-toxigenic variant of *C. botulinum* (Iida *et al.*, 1981). Various degrees of cross-reactivity between the two species have been revealed by immunofluorescence procedures (Walker & Batty, 1964; Hatheway & McCroskey, 1981) and by numerical taxonomy; for example, Kiritani *et al.* (1973) grouped 20 proteolytic strains of *C. botulinum* and 14 strains of *C. sporogenes* in the same phenon. Similarly Group III *C. botulinum* strains (i.e. types C and D) are indistinguishable from *C. novyi*. Type C *C. botulinum* can be cured of the prophage that codes for toxin production and converted by another phage to a *C. novyi* type A strain, and *C. botulinum* types C and D can be interconverted by phage (Eklund & Poysky, 1981).

Our extensive study of cross-reactive antigens in the genus *Clostridium* (Poxton & Byrne, 1984) demonstrated shared antigens between *C. botulinum* types A and B (proteolytic) and *C. sporogenes*. Moreover, antigens from types C and D cross-reacted with antiserum to *C. novyi* type A. The present study extends the investigation of these cross reactions.

Abbreviations: npB, type B non-proteolytic strain; pB, type B proteolytic strain; TBS, Tris-buffered saline.

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METHODS

Bacteria. All strains of *C. botulinum* were from the collection in the Botulinum Laboratory, Unilever Research, Sharnbrook, Bedford, UK: type A, strain nos 1, 7, 8, 9; type B (proteolytic, pB), strain nos 25, 26, 27; type B (non-proteolytic, npB), strain nos 188, 194, 229; type C, strain no. 205; type D, strain no. 154 and type E, strain nos 34, 118, 126, 250. The *C. novyi* strains NCTC 538 (type A), NCTC 9747 (type C) and NCTC 8350 (type D) and *C. sporogenes* NCTC 8594 were obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT, UK. Other strains from our laboratory culture collection were: *C. novyi* GR2A and GR3A (type A strains) and GR2B (type B), and *C. sporogenes* strains 24 and 33. The strains of *C. botulinum* were checked for toxin type by Mr C. Grimes of Unilever Research at the time of EDTA antigen preparation. The culture supernates were used in mouse toxin–antitoxin neutralization tests as outlined by Willis (1977). Neutralizing antitoxin was obtained from the Pasteur Institute, Paris, France. The identity of all strains was confirmed. The strains of *C. novyi* and *C. sporogenes* were fully characterized by the method of Holdeman *et al.* (1977).

Growth conditions. Stationary phase bacteria (0·2 ml) which had been cultured in Robertson's cooked meat broth and incubated anaerobically with 10% CO_2 for up to 48 h at 37 °C, were used to inoculate 100 ml proteose peptone/yeast extract broth (Holbrook *et al.*, 1977). Cultures were incubated anaerobically with 10% CO_2 for 16 h, or 40 h for *C. botulinum* types C and D, at 37 °C. Spore formation as judged by phase-contrast microscopy was negligible under these conditions.

Antigen extraction. Extraction with 10 mM-EDTA of washed bacteria from the 100 ml cultures was as previously described (Poxton & Byrne, 1981) except that the dialysis step was omitted after it was shown to be unnecessary.

Preparation of antisera. Antisera to *C. novyi* type A NCTC 538 and *C. sporogenes* NCTC 8594 were raised in New Zealand White rabbits by intravenous injection of whole, washed UV-killed bacteria over a 5 week period as detailed by Poxton (1979). A standard dose of 10^9 organisms per injection was used. These sera are referred to as reference species antisera.

Indirect enzyme-linked immunosorbent assay (ELISA). The EDTA extracts were diluted to 30 μg protein ml^{-1} in antigen buffer (50 mM-sodium carbonate buffer, pH 9·6) and 50 μl volumes were used to coat wells in flat-bottomed polystyrene microtitre plates. They were incubated for 4 h at 37 °C and overnight at 4 °C. After washing three times with 0·15 M-NaCl containing 0·05% (v/v) Tween 20 (Tween saline), doubling dilutions (1 in 400 to 1 in 102400) of rabbit antiserum in Tween PBS (50 mM-sodium phosphate buffer pH 7·4 containing 0·15 M-NaCl, 0·05% Tween 20 and 0·02% sodium azide) were added to each well (50 μl) and incubated for 4 h at room temperature. The plates were washed three times in Tween saline, and anti-rabbit IgG-alkaline phosphatase conjugate (Miles, Slough, UK) diluted 1 in 500 in Tween PBS was added to each well (50 μl) and incubated overnight at room temperature. After washing three times in Tween saline, 50 μl *p*-nitrophenyl phosphate (Sigma) at a concentration of 1 mg ml^{-1} in 50 mM-sodium carbonate buffer pH 9·8, containing 1 mM-$MgCl_2$, was added to each well. After incubation for 1 h at room temperature, the plates were read in a Titertek Multiskan (Organon Teknika, St Neots, UK): the highest dilution of serum that gave an $A_{405} > 1·0$ was recorded as the titre.

Polyacrylamide gel electrophoresis and electroblot transfer. SDS-polyacrylamide gel electrophoresis was done on 10% slab gels with the buffer system of Laemmli (1970) as described by Poxton & Brown (1979). Approximately 50 μg protein (based on the Lowry method) was run in each track.

Electroblot transfer was essentially by the method of Towbin *et al.* (1979). Material was transferred to nitrocellulose membrane (Transblot Transfer Medium, Bio-Rad) in Tris/glycine/methanol buffer, pH 8·3 over a 16 h period at 12 V, 40 mA. The transferred antigens were detected with the ImmunBlot immunoassay (Bio-Rad). Briefly, the membrane, after washing for 10 min in Tris-buffered saline (20 mM-Tris, 500 mM-NaCl, pH 7·5; TBS) was placed in 3% (w/v) gelatin TBS for 45 min to block any unbound sites. It was then transferred into the *C. sporogenes* or *C. novyi* antiserum diluted 1 in 200 in 1% gelatin TBS and incubated for 3 h at room temperature. After two 10 min washes in 0·025% Tween 20 TBS the membrane was placed into goat anti-rabbit IgG-horseradish peroxidase conjugate (Bio-Rad) diluted 1 in 3000 in 1% gelatin TBS and incubated for 1 h at room temperature. After a further two 10 min washes in Tween TBS it was placed into HRP colour development solution (Bio-Rad) which contains 4-chloro-1-napthol and hydrogen peroxide. Colour developed within 5–15 min. The reaction was stopped by placing into distilled water and washing with several changes of water over 1 h. All of the above steps were done in clean glassware and with gentle agitation throughout.

RESULTS

ELISA studies

EDTA extracts of all of the test strains were reacted in an ELISA system with our reference antisera to *C. sporogenes* and *C. novyi* (Table 1). All the proteolytic type B, three out of four of the type A and one type E strains reacted strongly with the *C. sporogenes* reference species

Table 1. *Reactions obtained with EDTA extracts of C. botulinum, C. sporogenes and C. novyi in ELISA with antisera to C. sporogenes and C. novyi*

The figures presented are typical of results obtained in repeated experiments. –, Titre of less than 400.

EDTA antigen prepared from:	Titre of reaction of the stated antigen with antiserum raised to whole cells of: *C. sporogenes* NCTC 8594	*C. novyi* NCTC 538
C. botulinum		
A1	1600	–
A7	3200	–
A8	400	–
A9	–	–
pB25	6400	–
pB26	400	–
pB27	800	–
npB188	–	–
npB194	–	–
npB229	–	–
C205	–	1600
D254	–	3200
E34	–	–
E118	6400	–
E126	–	–
E250	–	–
C. sporogenes		
NCTC 8494	6400	–
24	1600	–
33	1600	–
C. novyi		
A NCTC 538	–	12800
A GR2A	–	800
A GR3A	–	1600
B GR2B	–	1600
C NCTC 9747	–	1600
D NCTC 8350	–	1600

antiserum. The type A strain that did not react (A9) was not even a weak reaction; no colour was detectable by the ELISA plate reader at the lowest dilution (1 in 400) used. The two heterologous *C. sporogenes* strains reacted significantly with the reference *C. sporogenes* antiserum. No reaction was demonstrated between the non-proteolytic B, the C, the D, three out of four of the E strains and all of the *C. novyi* strains with the *C. sporogenes* antiserum.

With the *C. novyi* antiserum only *C. novyi* strains and *C. botulinum* strains of types C and D showed a reaction.

Polyacrylamide gel electrophoresis and electroblot transfer

Initially, EDTA extracts from one strain of each type of *C. botulinum* and from one strain each of *C. sporogenes* and *C. novyi* were run on 10% SDS-PAGE slab gels and stained with Coomassie blue (Fig. 1 *a*). All patterns were complex but there were marked similarities between those of *C. botulinum* types A and B (proteolytic) and those of types C and D (tracks 2 and 3, and 5 and 6 respectively).

Two duplicates of this gel (unstained) were electrophoretically transferred to nitrocellulose membranes and were probed with the *C. sporogenes* and *C. novyi* antisera. The results, after development with the horseradish peroxidase conjugate reagents, are shown in Fig. 1 (*b*, *c*). The antiserum to *C. sporogenes* (Fig. 1 *b*) reacted significantly with *C. botulinum* type A1 (track 2) and proteolytic type B25 (track 3) and the homologous *C. sporogenes* (track 8) EDTA extracts. In the cross-reaction with the *C. botulinum* proteolytic type B25 extract many strongly staining bands were visible, the overall intensity being stronger than with the homologous reaction. The cross-

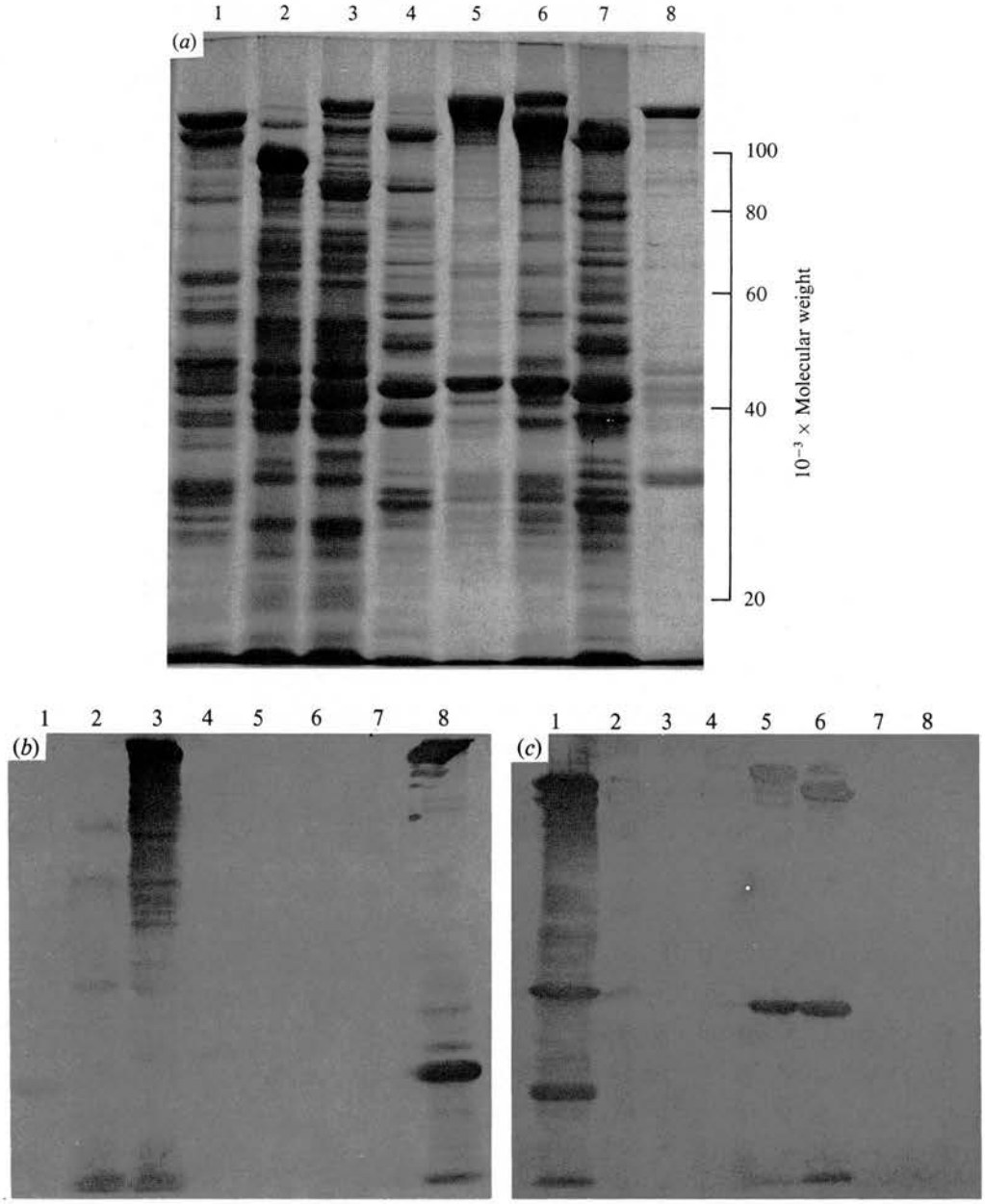

Fig. 1. SDS-polyacrylamide gel electrophoresis and corresponding electroblot transfers of EDTA extracts of strains of *C. botulinum*, *C. novyi* and *C. sporogenes*. Approximately 50 µg protein in 50 µl sample buffer were run on each track of a 10% acrylamide gel. Track 1, *C. novyi* type A NCTC 538; track 2, *C. botulinum* A1; track 3, *C. botulinum* pB25; track 4, *C. botulinum* npB188; track 5, *C. botulinum* C205; track 6, *C. botulinum* D154; track 7, *C. botulinum* E34; track 8, *C. sporogenes* NCTC 8594. (*a*) Coomassie blue-stained gel. (*b*) Electroblot transfer of (*a*) reacted with antiserum to *C. sporogenes* NCTC 8594. (*c*) Electroblot transfer of (*a*) reacted with antiserum to *C. novyi* NCTC 538.

reaction with the type A extract was less strong but several bands, which were common to the type B, were stained. Single faint cross-reacting bands were also apparent in the *C. novyi* and the *C. botulinum* non-proteolytic type B extracts. The antiserum to *C. novyi* (Fig. 1*c*) only cross-reacted with extracts from *C. botulinum* types C and D, one major antigen being present in each.

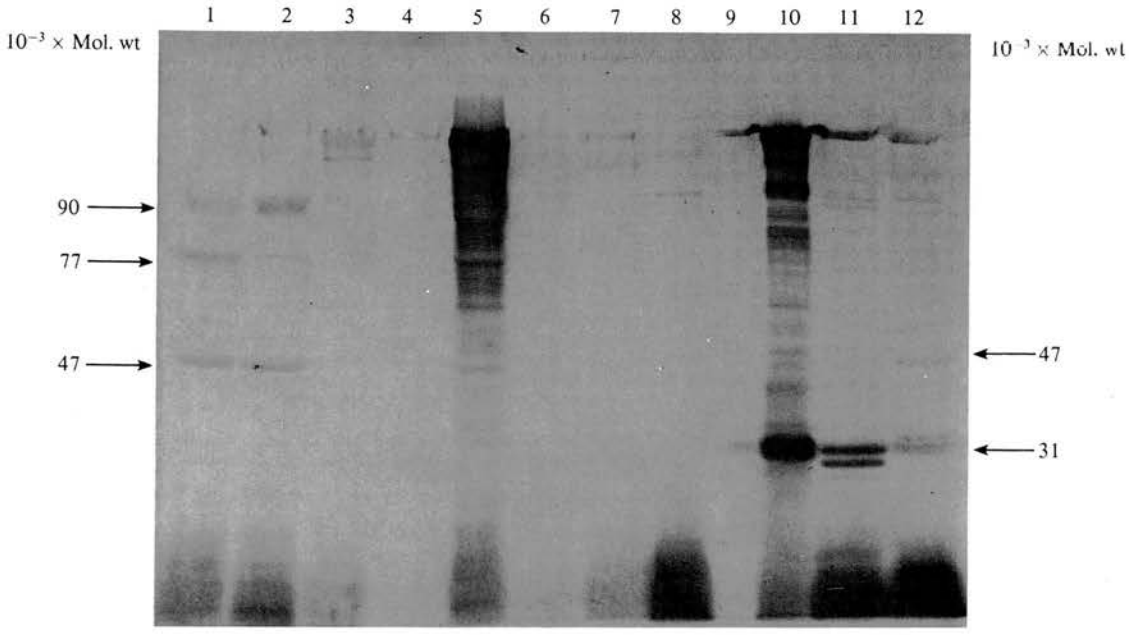

Fig. 2. Electroblot transfers of EDTA extracts of strains of *C. botulinum* and *C. sporogenes* separated as in Fig. 1 (*a*) and reacted with antiserum to *C. sporogenes* NCTC 8594. Track 1, *C. botulinum* A1; track 2, *C. botulinum* A7; track 3, *C. botulinum* A8; track 4, *C. botulinum* A9; track 5, *C. botulinum* pB25; track 6, *C. botulinum* pB26; track 7, *C. botulinum* pB27; track 8, *C. botulinum* E118; track 9, blank; track 10, *C. sporogenes* NCTC 8594; track 11, C. *sporogenes* 24; track 12, *C. sporogenes* 33.

In order to demonstrate fully the cross-reactions revealed by ELISA between *C. botulinum* strains and the two antisera and also to compare these inter-species cross-reactions with the reactions within *C. sporogenes* and *C. novyi* strains, two further electroblot transfers were done. EDTA extracts of all the strains of type A and proteolytic B, strain E118 (see Table 1) of *C. botulinum* and the three *C. sporogenes* strains were run together on an SDS polyacrylamide gel. They were transferred to nitrocellulose and reacted with the *C. sporogenes* antiserum (Fig. 2). Extracts from A1 and A7 (tracks 1 and 2) showed almost identical patterns with a series of diffusely staining bands at the bottom of the gel and three common bands at molecular weights of approximately 90000, 77000 and 47000. The A8 extract (track 3) was similar to A1 and A7 except that the bands were weaker in staining intensity and some high molecular weight bands were faintly visible at the top of the gel. The proteolytic type B strains (tracks 5–7) produced a much more variable reaction than the type A strains. The extract from B25 (track 5) produced a very complex pattern of strongly staining bands as already described. Reactions with extracts from B26 and B27 were much weaker but the patterns produced were similar to each other. A significant reaction was also produced by the extract from strain E118 (track 8). A series of strongly staining low molecular weight bands at the front of the gel was the main feature of this pattern with a few faint higher molecular weight bands. When compared with the homologous reaction (track 10) the two other strains of *C. sporogenes* produced much less complex patterns (tracks 11 and 12). Again the series of low molecular weight bands was common, while there were other common bands with molecular weight of approximately 47000 and 31000.

Similarly the type C and D strains of *C. botulinum* and all the *C. novyi* strains were run together and reacted with the *C. novyi* type A antiserum (Fig. 3). The *C. botulinum* types C and D (tracks 1 and 2) both produced similar patterns. A band with a molecular weight of approximately 47000 was the major cross-reacting antigen, but several high molecular weight bands were apparent at the top of the gel and there was a series of regularly spaced cross-reacting low molecular weight bands at the bottom of the gel. The reactions between the three type A *C. novyi* strains were very

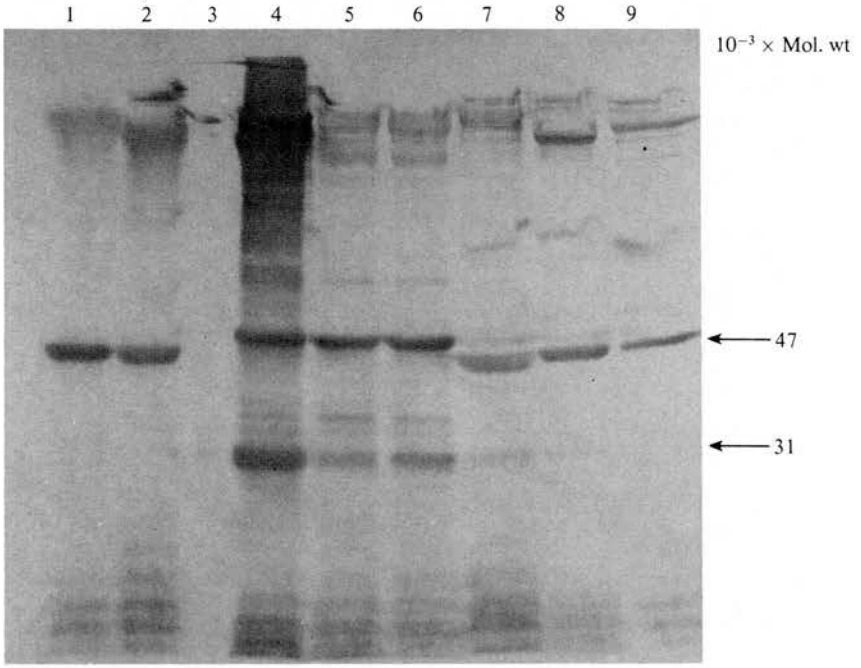

Fig. 3. Electroblot transfers of EDTA extracts of strains of *C. botulinum* and *C. novyi* separated as in Fig. 1 (*a*) and reacted with antiserum to *C. novyi* type A NCTC 538. Track 1, *C. botulinum* C205; track 2, *C. botulinum* D154; track 3, blank; track 4, *C. novyi* A NCTC 538; track 5, *C. novyi* A GR2A; track 6, *C. novyi* A GR3A; track 7, *C. novyi* B GR2B; track 8, *C. novyi* C NCTC 9747; track 9, *C. novyi* D NCTC 8350.

similar to each other (tracks 4, 5 and 6). The major cross-reacting antigens had molecular weights of 48 000 and 31 000 with a series of regularly spaced bands at the bottom of the gel. The *C. novyi* types B, C and D produced similar patterns (tracks 7, 8 and 9): there was a major band with a molecular weight of 47 000 and a series at the bottom of the gel which was similar to all the other tracks on the gel.

DISCUSSION

The results obtained with the ELISA and the electroblot procedures agree closely and show conclusively that most strains of *C. botulinum* types A and proteolytic B (Group I) share antigens with *C. sporogenes*, whereas types C and D (Group III) share antigens with *C. novyi*. This provides further evidence of the close taxonomic relationships between these species. In view of the limited number of strains investigated, it is not yet certain how widely these cross-reactions occur and how many exceptions there may be to the general rule demonstrated here. For example, one of the strains of *C. botulinum* type A did not react, whilst one of the type E strains did react with *C. sporogenes* antiserum. Moreover, varying degrees of cross-reactivity could be demonstrated with the three type A and three proteolytic type B strains that cross-reacted with the *C. sporogenes* antiserum. This was also true for the three strains of *C. sporogenes* investigated, and indicates antigenic heterogeneity. Reactions within the *C. novyi* strains were much more uniform. No major differences were seen between the three *C. novyi* type A strains. The *C. novyi* strains of types B, C and D showed close antigenic similarity with each other and with the *C. botulinum* strains of types C and D.

The patterns of cross-reactivity revealed by electroblot transfer all showed some similarities. Most of the major cross-reactive bands corresponded to predominant protein bands on the Coomassie blue-stained gels. All strains that reacted with antiserum, however, also showed

patterns of regularly spaced bands at the bottom of the gels. These bands did not appear to correspond to any of the protein bands on the Coomassie blue-stained gels. They were reminiscent of the patterns produced by lipopolysaccharides of Gram-negative bacteria when stained with silver (Tsai & Frasch, 1982). It is possible that these cross-reacting antigens were lipocarbohydrate molecules from the cytoplasmic membrane (lipoteichoic acid analogues). We know that, at least for *C. difficile* and *C. sordelli*, the membrane lipocarbohydrates are cross-reacting antigens and that they are solubilized by EDTA treatment (Poxton & Cartmill, 1982).

There was no attempt made in this study to use antiserum raised to any *C. botulinum* strain. The aim was to use antisera raised against cells that were as close as possible to living organisms: washing and killing with a minimum dose of UV light were the only treatments used. Obtaining undenatured cells of toxigenic *C. botulinum* devoid of toxin is not possible. No attempt was made to work with *C. botulinum* types F and G as the author was not protected against these types.

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Detection of *Clostridium difficile* Toxin by Counterimmunoelectrophoresis: a Note of Caution

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Recent methods for detection of *Clostridium difficile* toxin by counterimmunoelectrophoresis might lead to errors. False-positives may be attributable to soluble cell surface antigens reacting with impure antitoxin.

Several workers have recently attempted to develop a counterimmunoelectrophoresis (CIE) method for the detection and quantitation of *Clostridium difficile* cytotoxin in both fecal and culture filtrates (3, 4). If valid and successful, such a system would have obvious advantages over the cytotoxic assays that are currently in use for investigation of antibiotic-associated colitis. We are convinced, however, that the antigen that is being detected by CIE and reported in these two papers is not necessarily the *C. difficile* toxin and could be a cell surface antigen of *C. difficile*. Our evidence for this is as follows.

(i) We have recently shown that *C. difficile*, *Clostridium sordellii*, and *Clostridium bifermentans*, but no other clostridial species tested, have at least one surface carbohydrate antigen in common (1). Welch et al. (4) show that cytotoxic assay-negative strains of *C. sordellii* and *C. bifermentans* are both positive by CIE. Similarly, in both papers (3, 4) all *C. difficile* strains tested are CIE positive, although some are cytotoxic assay negative.

(ii) Although we are unable to test any of the conditions available in the United States, our own *C. difficile* antitoxin and the *C. sordellii* antitoxin supplied by the Wellcome Research Laboratories (Beckenham, England) have antibodies to the common cell surface antigens. Commercial preparations of antitoxin are usually raised against relatively impure toxin preparations.

(iii) In attempting to purify the *C. difficile* toxin by the method of Rolfe and Finegold (2) we found that the cell surface carbohydrate antigen copurified with the toxin.

(iv) *C. difficile* in culture, especially if any cell lysis has occurred, releases much cell surface antigen into the medium.

While agreeing with the other authors (3, 4) as to the importance of identification of toxigenic strains of *C. difficile* for the management of cases of antibiotic-associated colitis, we suggest the need for caution in interpreting the results of CIE studies in this area. The absorption of the antitoxin with whole cells of *C. sordellii*, *C. bifermentans*, or *C. difficile* will remove the anti-surface immunoglobulins and may allow more specific results. Similarly, antitoxin raised against absolutely pure toxin will give specific results.

There is still much that is not understood about the roles of both toxigenic and nontoxigenic strains of *C. difficile* as enteropathogens; possibly erroneous reports of toxin-positive strains will increase our confusion.

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Immunochemistry of the Cell-Surface Carbohydrate Antigens of *Clostridium difficile*

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Two carbohydrate cell-surface antigens were extracted from *Clostridium difficile*. One was extracted from pure cell walls by NaOH and contained glucose, mannose, galactosamine and phosphate in the approximate molar proportions of 2:0·65:1:0·63. The other antigen was extracted with phenol from the disrupted contents of whole cells and purified by chromatography on Sepharose 6B and an immunoabsorbent column; it contained glucose, glucosamine, phosphate and fatty acid in the approximate molar proportions of 2:1:1·6:0·04. Both antigens showed partial immunological identity and both cross-reacted with *Clostridium sordellii* antiserum. They are analogues of the wall and membrane teichoic acids of other Gram-positive bacteria.

INTRODUCTION

Clostridium difficile is a Gram-positive, motile, spore-forming, rod-shaped bacterium which, until recently, was regarded as an uncommon, non-pathogenic organism. In 1977 it was shown to be involved in the aetiology of antibiotic-associated colitis; it may also be involved in a significant number of antibiotic-associated diarrhoeas (Larson, 1979; Bartlett, 1979). A recent analysis of the EDTA-soluble antigens of this and related species by Poxton & Byrne (1981) showed that a carbohydrate antigen is shared by *C. difficile* and *C. sordellii*. The present study investigates the cell-surface carbohydrate antigens of *C. difficile* with a view to showing the location and nature of the common *C. sordellii*/*C. difficile* antigen, and also reports the first investigation of the secondary cell wall polymer (teichoic acid or analogue) and of the membrane lipocarbohydrate polymer (lipoteichoic acid or analogue) of a *Clostridium* species.

METHODS

Culture of organism and preparation of cell walls. Clostridium difficile NCTC 11223 was cultivated in 16-litre batches in an L.H. fermenter (L.H. Engineering, Stoke Poges, Berks.). Oxygen-free nitrogen was bubbled at the rate of 300 ml min^{-1} through PPY medium (Holbrook *et al.*, 1977) which contained 0·4% (w/v) sodium carbonate and 0·75% (w/v) cysteine hydrochloride, for 16 h at 37 °C. The medium was inoculated with 100 ml of a 16 h culture of *C. difficile* in PPY medium and incubated for 18 h at 37 °C with nitrogen bubbling. Sporulation was negligible under these conditions. Bacteria were harvested by centrifugation at 2000 ***g*** for 40 min and washed once in phosphate-buffered saline (PBS: 50 mM-phosphate buffer pH 7·5 containing 0.15 M-NaCl). The wet pellet was suspended to a concentration of approximately 30% (w/v) in PBS and the bacteria were disrupted by passage through a French Pressure Cell (Aminco, American Instrument Co. Inc., Silver Springs, Md., U.S.A.) at 6000–7000 lbf in^{-1} (41–48 MPa). The suspension was centrifuged at 45000 ***g*** for 20 min at 4 °C. The supernate was removed, freeze-dried and retained for subsequent extraction of membrane antigen. The upper white layer of cell walls was carefully removed from the small pellet of unbroken cells, suspended in a small volume of water and heated to 80 °C for 5 min to inactivate the autolytic enzymes; it was then purified by treatment with 2·5% (w/v) sodium dodecyl sulphate (SDS) as described by Poxton *et al.* (1978).

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Extraction of cell-wall antigen. Freeze-dried SDS-treated cell walls were extracted with 0·5 M-NaOH for 4 h at 20 °C by the method of Archibald *et al.* (1969). The NaOH was neutralized with dilute HCl and salts were removed by extensive dialysis against distilled water. The extract was freeze-dried.

Extraction of membrane antigen. The freeze-dried supernate (8 g) obtained after a cell breakage was defatted with 2 × 200 ml chloroform/methanol (2 : 1, v/v), over 24 h, extracted with cold 80% (w/w) phenol and partially purified on a Sepharose 6B (Pharmacia) column (65 × 1·6 cm) by the method of Coley *et al.* (1975).

Preparation of antisera. Antisera against u.v.-killed, whole, washed cells of *C. difficile* NCTC 11223 and *C. sordellii* NCTC 8780 were prepared as described previously (Poxton & Byrne, 1981).

Immunoelectrophoresis. Crossed immunoelectrophoresis (CIE) was performed by the procedure of Weeke (1973) as described for *C. difficile* by Poxton & Byrne (1981). Fused rocket immuno-electrophoresis (FRIE) was done by the method of Svendsen (1973), with the same buffer system as used for CIE.

Preparation of immunoabsorbent column. Immunoglobulin G (IgG) was prepared from 3 ml antiserum to *C. difficile* NCTC 11223 by batch treatment with DEAE-cellulose (Whatman DE 52) by the method of Hudson & Hay (1976). IgG (50 mg) was coupled to 10·5 ml swollen CNBr-activated Sepharose 4B (Pharmacia) by the method recommended by Pharmacia in 'Affinity Chromatography, Principles and Methods'. The immunoabsorbent was packed in a column (10 × 1 cm) and equilibrated for storage in 0·1 M-phosphate buffer pH 7·0 containing 0·5% (v/v) Tween 80 and 0·01% (w/v) Merthiolate (thimerosal).

Analytical techniques. Phosphorus was estimated by the method of Chen *et al.* (1956); sugars, as glucose equivalents, were estimated by the method of Dubois *et al.* (1956); sialic acid was estimated by the method of Aminoff (1961).

Paper chromatography. Acid hydrolysates of carbohydrates (2 M-HCl, 100 °C, 2 h) were dried several times *in vacuo* over NaOH pellets and phosphorus pentoxide. They were chromatographed on Whatman no. 1 paper in solvent A [butan-1-ol/pyridine/water (6 : 4 : 3, by vol.)] for reducing sugars and stained with the alkaline silver nitrate reagents (Trevelyan *et al.*, 1950) and in solvent B [propan-1-ol/aqueous ammonia (S.G. 0·88)/water (6 : 3 : 1, by vol.)] for polyols and stained with the periodate–Schiff's reagents (Baddiley *et al.*, 1956).

Gas chromatography. (*a*) *Sugars.* Dry hydrolysates of carbohydrates were converted to alditol acetates by a method modified from that of Lindberg *et al* (1978). The hydrolysate (1–20 mg) was dissolved in 1 ml distilled water, and 10 mg sodium borohydride was added. After 1 h at 20 °C, excess borohydride was destroyed by the addition of a few drops of glacial acetic acid. After drying by rotary evaporation, borate, as methyl borate, was removed by three distillations with methanol. The dry alditols were acetylated in 1 ml pyridine/acetic anhydride (1 : 1, v/v) at 100 °C for 1 h. Excess reagents were removed by several co-distillations with toluene in a rotary evaporator, and the alditol acetates were taken up in 1 ml chloroform, washed twice with water, dried by rotary evaporation and finally dissolved in 0·5 ml chloroform. Samples (1 μl) were examined on columns of 3% OV225 on Gas-chrom Q (for amino sugar derivatives) and 3% SP2330 on Supelcoport (for pentose and hexose derivatives) in a Pye-Unicam model 104 gas chromatograph, with the temperature controlled between 190 °C and 240 °C by 2 °C min^{-1}.

(*b*) *Fatty acids.* Membrane antigen which had been eluted from the immunoabsorbent column was dried and methanolysed by heating with 0·5 M-HCl in methanol (1 ml) in a sealed vessel for 2 h at 65 °C. Water (2 ml) and ether (4 ml) were added; after mixing, the phases were separated. The ether phase was brought to dryness in a rotary evaporator, redissolved in ether (0·5 ml) and examined for methyl esters of fatty acids on a column of 3% SP2330 on Supelcoport at 150 °C.

RESULTS

Cell wall antigen

From 50 mg lyophilized cell walls, 20 mg was solubilized by 0·5 M-NaOH. When this wall antigen extract was examined by crossed-immunoelectrophoresis (CIE) against antiserum to whole cells of *C. difficile* NCTC 11223, one precipitin line was observed (Fig. 1*a*). This line did not correspond to any of the lines produced by the EDTA extract of whole cells (see Poxton & Byrne, 1981) but it did correspond to one produced by an autolysate of whole cells of *C. difficile* (Fig. 1*b*). When this wall antigen was run against antiserum to *C. sordellii* NCTC 8780 in CIE, an identical precipitin line was produced.

Chemical analysis of the wall antigen showed it to contain sugar (phenol/sulphuric acid assay method) and phosphorus in the molar ratio of 4·2 : 1. Analysis of the constituent sugars by paper chromatography of acid hydrolysates showed the presence of glucose, mannose and galactosamine. No ribitol or glycerol was detected. Gas chromatography of alditol acetate derivatives showed the presence of the same three sugars in the molar ratio

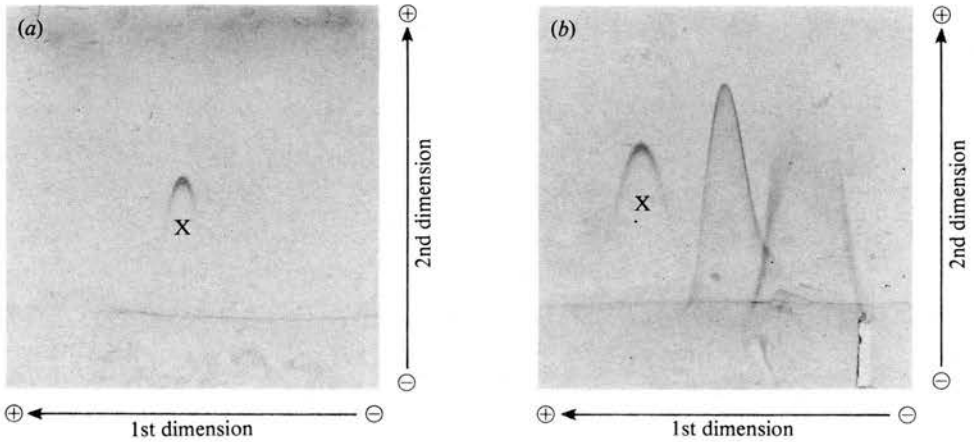

Fig. 1. Crossed immunoelectrophoresis of (*a*) NaOH-soluble cell-wall antigen (6 µl of 5 mg ml^{-1} solution) and (*b*) soluble fraction of autolysed whole cells of *C. difficile* (6 µl of supernate from cells allowed to autolyse in PBS for 24 h at 37 °C) run against 65 µl *C. difficile* NCTC 11223 antiserum. The precipitin line X is common to both preparations.

glucose : mannose : galactosamine of 2 : 0·65 : 1. As galactosamine does not react in the phenol/sulphuric acid assay this suggests a ratio of total sugar : phosphorus of 6 : 1 or glucose : mannose : galactosamine : phosphorus of 2 : 0·65 : 1 : 0·63. Sialic acid was absent. The polymer (1 mg) was treated with 4 units of phosphomonoesterase (Boehringer, Grade I) for 3 h at 37 °C in 0·5 ml 0·05 M-ammonium carbonate pH 9·5 under toluene. All the phosphate was shown to have been present in the monoester form. The dephosphorylated product and the untreated control gave an identical precipitin line when run in CIE against *C. difficile* antiserum.

Membrane antigen

The crude phenol extract of the lyophilized supernate from the broken cells was run in CIE against *C. difficile* whole-cell antiserum. Two precipitin lines were present and they appeared to be joined (Fig. 2). After fractionation on a Sepharose 6B column, fractions were analysed for carbohydrate, phosphate and nucleic acid and by fused-rocket immunoelectrophoresis (Fig. 3). The fractions corresponding to the two antigens were pooled and each was examined against *C. difficile* whole-cell antiserum by tandem CIE with an EDTA extract of whole cells of *C. difficile* NCTC 11223 (Poxton & Byrne, 1981) and with the wall antigen according to the method of Krøll (1973). The antigen that eluted from the Sepharose 6B column in the void volume (Peak I) gave a precipitin line that was identical with line 2 in the EDTA extract, i.e. the carbohydrate antigen that is common to *C. difficile* and *C. sordellii*. The antigen that was included by the Sepharose column (Peak II) gave a precipitin line that was identical with the purified wall antigen.

The material in Peak I contained one antigen, but as there was extensive contamination with nucleic acid, further purification was necessary before a chemical analysis could be made. The immunoabsorbent column, prepared as described in Methods, was equilibrated in borate-buffered saline (BBS: 0·1 M-NaCl, 0·2 M di-sodium tetra-borate, pH 7·3). This was to remove any phosphate buffer that might subsequently contaminate the eluted antigen. The material from Peak I (2·5 mg) was dissolved in BBS, applied to the column and washed with 20 ml BBS. The flow was reversed and the antigen was eluted with sodium carbonate buffer pH 9·6/ethylene glycol (1 : 1, v/v). Ten 4 ml fractions were collected and screened for antigen by fused-rocket immunoelectrophoresis. The fractions containing antigen were pooled and concentrated to 2 ml in an Amicon (Lexington, Mass., U.S.A.) ultrafiltration cell (PM10

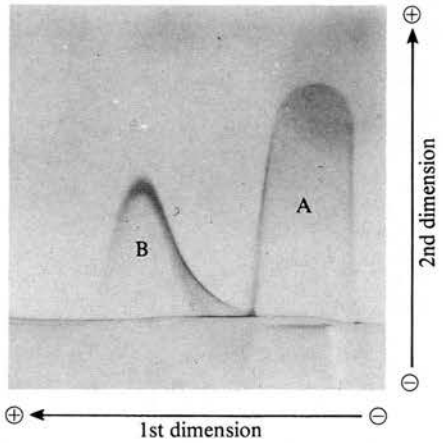

Fig. 2. Crossed immunoelectrophoresis of phenol extract of *C. difficile* cell contents (6 µl of 5 mg carbohydrate ml^{-1} solution) run against 65 µl *C. difficile* antiserum. Lines A and B correspond to the antigens separated in Fig. 3.

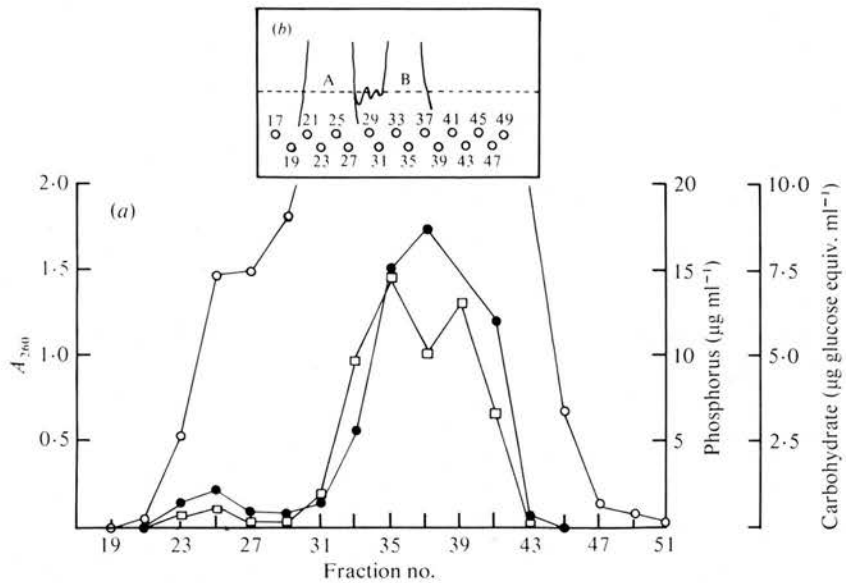

Fig. 3. (*a*) Purification of phenol extract of *C. difficile* cell contents run on a Sepharose 6B column (60 × 1·6 cm). Fractions (3 ml) were collected and screened for nucleic acid at 260 nm (○); 0·1 ml of each fraction was screened for phosphorus (●) and carbohydrate (□).

(*b*) Drawing of fused rocket immunoelectrophoresis of fractions run against antiserum to *C. difficile* NCTC 11223. Lines A and B correspond to the same lines in Fig. 2.

membrane). Paper chromatography of acid hydrolysates showed the presence of glucose and glucosamine. No glycerol or ribitol was detected. Gas chromatography showed the presence of the same sugars in the molar ratio glucose : glucosamine of 2 : 1. After methanolysis (see Methods) the ether phase was analysed by gas chromatography and the fatty acids were shown to consist of C16:0, 25·1%; C16:1, 7·1%; C18:0, 8·6%; C18:1, 43·8% and

C18:2, 15·3%. Percentages were calculated by measuring the area of the peaks of the gas-chromatography trace. Total fatty acid was calculated by comparison with methyl stearate. Phosphate and carbohydrate (phenol/sulphuric acid) were estimated on the aqueous phase of the methanolysate. Results showed the overall composition of the antigen to be glucose, glucosamine, phosphate and fatty acid in the molar proportions 2:1:1·6:0·04.

Treatment of the membrane antigen with mild alkali (5 mM-lysine, 140 mM-KCl, pH 11·5) for 2 h at 20 °C released the lipid moiety for the carbohydrate portion, which then became indistinguishable from the wall antigen in crossed immunoelectrophoresis.

DISCUSSION

Clostridium difficile possesses two carbohydrate cell-surface antigens. The secondary cell wall polymer or teichoic acid analogue is a major component of the cell wall (40% by weight of SDS-purified walls). It was not released by EDTA but was solubilized during autolysis. This is similar to the behaviour of teichoic acids, which appear to be released but not degraded by autolytic enzymes (Duckworth, 1977). Its composition is unusual but it resembles the wall polymer described by Partridge *et al.* (1971), which was found in the walls of a *Micrococcus* species. After extraction with alkali all the phosphorus was present as monoester and its removal by phosphomonoesterase did not alter its antigenic properties in crossed immunoelectrophoresis. This means that the phosphorus is not part of the antigenic determinant; as structural determinations have not been possible, the location of the phosphorus is as yet unknown.

The other carbohydrate polymer was extracted by methods developed for the isolation of membrane or lipoteichoic acids. The material excluded by Sepharose 6B and further purified by immunoabsorbent chromatography was the polymer analogous to the membrane teichoic acid. Its composition is unlike any polymer previously described. It is the common *C. difficile*, *C. sordellii*/*C. bifermentans* EDTA-soluble carbohydrate antigen (Poxton & Byrne, 1981) which was released due to the loss of membrane integrity following chelation of divalent cations by EDTA. The antigen in Peak II from the Sepharose column was immunologically identical to the wall carbohydrate antigen. This agrees with the observation of Coley *et al.* (1975) that the wall teichoic acids of several Gram-positive bacteria are often found in Peak II. When the lipocarbohydrate was deacylated with mild alkali, the carbohydrate fraction became immunologically identical to the wall polymer. It is therefore reasonable to ask whether the antigenic material in Peak II from the Sepharose 6B column is wall antigen or deacylated membrane antigen.

As both wall and membrane carbohydrate share immunological determinants, and both react with *C. sordellii* antiserum, they cannot be exploited for the detection of *C. difficile* by immunological means. A specific serological system for detection of *C. difficile* and distinction from *C. sordellii* would require an antiserum devoid of antibodies directed against either wall or membrane carbohydrate antigens.

C. difficile thus appears similar to aerobic Gram-positive bacteria in possessing a secondary cell wall carbohydrate and a membrane-bound lipocarbohydrate. These polymers are not strict teichoic acids as they do not contain ribitol or glycerol phosphate. They are, however, phosphate-containing carbohydrates. Their composition is more complex than any related phosphorylated carbohydrates yet described.

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DETECTION AND ISOLATION OF *CLOSTRIDIUM DIFFICILE*

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SUMMARY

Methods are described for the detection of *Clostridium difficile* cytotoxin in faecal extracts by tissue culture methods, and isolation of the organism on selective medium. Characterization steps include microscopy and biochemical tests, supplemented with gas chromatography of metabolic products. Biotyping and serotyping have possible epidemiological applications. New approaches for the detection of the organisms include the development of direct immunofluorescence assays and the determination of enterotoxic activity.

INTRODUCTION

Clostridium difficile is a Gram-positive, anaerobic, spore-forming, rod-shaped bacterium. Until recently, it was regarded as a commensal occurring in the faeces of 40–50% of neonates, but rarely in adults. It has been detected with increasing frequency since 1977, when it was shown to have a causative role in pseudomembranous colitis (PMC), and subsequently in antibiotic-associated colitis (AAC). It appears to be involved in a spectrum of conditions ranging from AAC through to antibiotic-associated diarrhoea (AAD), and also in exacerbations of chronic inflammatory bowel disease, post-operative diarrhoea and non-antibiotic-associated diarrhoea (Anonymous, 1981).

Our increasing awareness of the range of circumstances in which *C. difficile* can be found has been greatly advanced by the development of a highly selective medium (CCFA medium) by George *et al.* (1979). The development of an assay for *C. difficile* cytotoxin, the effect of which can be neutralized by *Clostridium sordellii* antitoxin, has allowed us to detect the presence of toxigenic organisms in faecal filtrates by observing a cytopathic effect on tissue monolayers.

The epidemiology and pathogenic mechanisms of *C. difficile* are still poorly understood. Animal studies and the clustering of some cases suggest that cross-infection can occur. The production of cytotoxin is circumstantially associated with severity of disease, but organisms that do not produce cytotoxin have been associated with enteropathies. Many questions have still to be answered. Only when *C. difficile* can be routinely detected, isolated and typed, and the nature of its extracellular products defined, can some of these questions be answered.

This paper reviews the methods currently available and others that are being developed for the detection, isolation, characterization and typing of *C. difficile*, and for the detection of its cytotoxin and presumptive enterotoxin (see below).

METHODS AND RESULTS

1. *Detection of cytotoxin*

(a) *Tissue culture assay for cytotoxin.* Since Larson *et al.* (1977) first described a heat-labile cytotoxin in the stools of patients with AAC which could be neutralized *in vitro* by *C. sordellii* antitoxin (Chang *et al.*, 1978; Rifkin *et al.*, 1978), tissue culture assays have been developed for the detection of *C. difficile* cytotoxin. Bartlett (1979) has reviewed the methods available—a summary of our own method is given here.

A liquid stool, or the homogenate of a solid stool in physiological saline, is centrifuged at 27,000 *g* for 10 min. Four-fold serial dilutions of the supernate are prepared in physiological saline and 10 μl volumes are added to 90 μl of Eagle's modified maintenance medium containing penicillin (200 units/ml), streptomycin (200 μg/ml), gentamicin (10 μg/ml) and amphotericin B (20 μg/ml) over monolayers of human embryo fibroblasts in Microtitre plates. The titre of cytotoxin is the highest dilution that produces complete rounding of more than 50% of the cells after incubation for 24 h at 37°C. Neutralization is performed by *C. sordellii* antitoxin (obtained from Dr. P. D. Walker of Wellcome Research Laboratories, Beckenham, U.K.). Antitoxin (10 μl of a 1 in 25 dilution) is added to a duplicate series of wells. A positive control, either a known positive stool extract or a purified *C. difficile* toxin, should be included. In our hands, there is no loss in potency if the toxin control is frozen and thawed several times.

There seems to be little correlation between cytotoxin titre and severity of disease. Although Burdon *et al.* (1981) have shown a significant correlation between a titre of greater than 6400 and pseudomembrane formation. It may be that the duration of exposure to the cytotoxin is a more valid criterion, but severity of the disease is best judged on clinical grounds. See, however, note on presumptive enterotoxin.

(b) *Other methods for detecting toxin.* Two groups (Welch *et al.*, 1980; Ryan *et al.*, 1980) have recently described a counter-immunoelectrophoresis method for detection of *C. difficile* toxin. We are certain, however, that this technique will result in many false-positives by the detection of a soluble cell-surface antigen common to *C. difficile*, *C. sordellii* and *C. bifermentans*. This technique should be used with the utmost caution (Poxton and Byrne, 1981a).

2. *Isolation of C. difficile*

Since the development of the highly selective cycloserine, cefoxitin, fructose agar (CCFA) medium by George *et al.* (1979), this has become the medium of choice for the isolation of *C. difficile*. It exploits the resistance of *C. difficile* to *D*-cycloserine (500 μg/ml) and cefoxitin (16 μg/ml) and its ability to ferment fructose. In our laboratory, and in many others, this medium has proved invaluable. After 24 h incubation at 37°C in anaerobic conditions, *C. difficile* produces a large (5 mm) flat to low umbonate yellow colony with a ground-glass appearance and a slightly filamentous edge. The selectivity of this medium is such that only after 24 h or 48 h incubation do other colonies appear. They are usually small, white and raised; these are usually lactobacilli and cannot be confused with the typical *C. difficile* colonies. In pilot studies, we have found no difference in isolation rates from faeces whether we use an anaerobic cabinet or bench technique. In quantitative platings from pure cultures we find that CCFA and blood agar give approximately equal yields. We do, however, find that colonies on CCFA are extremely sensitive to oxygen and should be sub-cultured without delay.

Hafiz and Oakley (1976) utilized the organism's resistance to *p*-cresol and developed a

selective medium containing 0.2% *p*-cresol. George *et al.* (1979), however, found that 0.2% *p*-cresol in reinforced clostridial agar inhibited all 16 of their stock strains, and concentrations below 0.2% were not selective.

3. *Characterization of the organism*

(a) *Microscopy.* In the light microscope *C. difficile* appears as a large (4–8 μm × 0.5–1 μm) Gram-positive rod with non-bulging sub-terminal to terminal spores. Under phase-contrast it exhibits a characteristic oscillating motility. In the electron microscope it is seen to have a rather sparse arrangement of peritrichous flagella.

(b) *Biochemical tests.* Most strains of *C. difficile* ferment glucose, fructose and mannitol; they hydrolyse aesculin and some produce gelatinase. All are indole and lecithinase negative. Some of our strains vary markedly in their biochemical reactions and this is summarized below. All strains can be conclusively characterized by gas chromatography (GC) of their volatile fatty acid metabolic products after culture in PPYSG medium (Deacon *et al.*, 1978). A typical GC profile together with an atypical profile from an asaccharolytic strain is shown in Fig. 1. The atypical profile could, however, not be

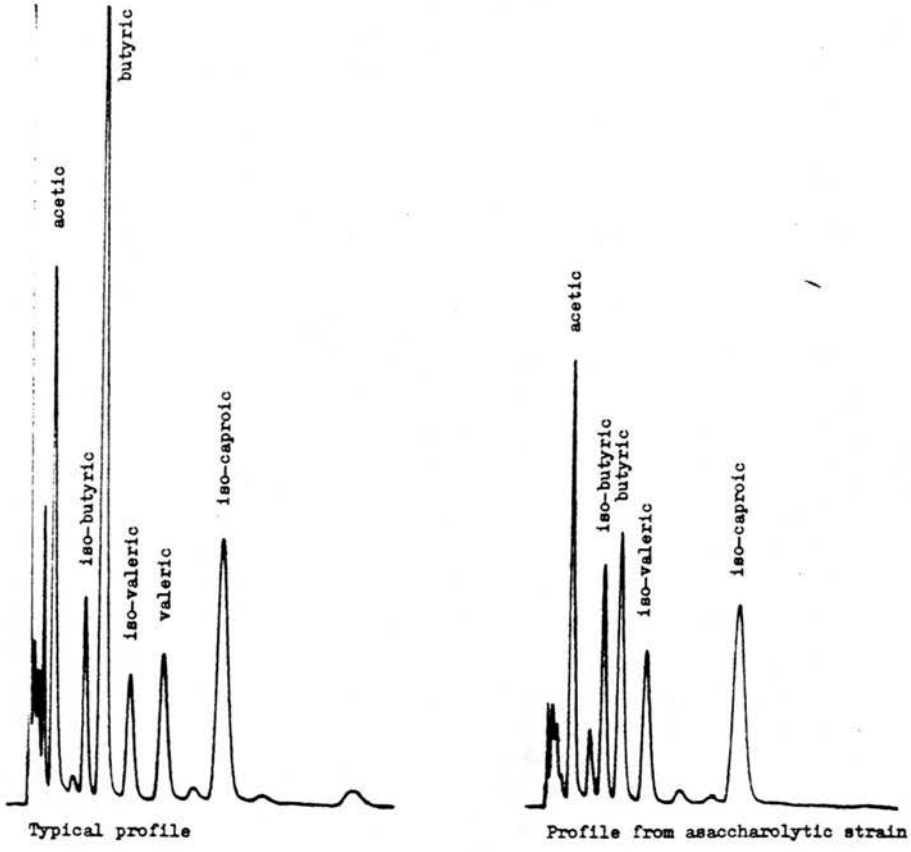

Fig. 1. Gas chromatograms of volatile fatty acid metabolic products from 18 h PPYSG cultures of a typical and an asaccharolytic strain of *C. difficile* on columns of SP 1220.

confused with any other *Clostridium* species. The significant peaks of acetic, butyric, iso-butyric, iso-valeric and iso-caproic acids are diagnostic.

Phillips and Rogers (1981) have recently developed a gas-chromatographic method for rapid identification of *C. difficile*. They used a modified CCFA medium supplemented with *p*-hydroxyphenyl acetic acid. This substrate is metabolized by *C. difficile* to *p*-cresol. *P*-cresol is assayed by extracting it from the agar below several presumptive colonies into ether and injecting the extract into a gas chromatograph. Results appear promising.

4. *Typing of C. difficile*

(a) *Biotyping*. We have investigated the biochemical properties of 43 of our isolates of *C. difficile*. These strains can be divided into seven different biotypes based on their reactions in six biochemical tests (Table 1). When several isolates were made from the same patient,

Table 1. Results of biochemical tests performed on 43 isolates of *C. difficile*

Test	Biotype						
	1	2	3	4	5	6	7
Aesculin hydrolysis	+	+	+	+	−	+	−
Glucose fermentation	+	+	+	+	+	−	−
Fructose fermentation	+	+	+	+	+	+	−
Mannitol fermentation	+	+	+	+	+	+	−
Mannose fermentation	+	+	−	−	−	−	−
Gelatinase production	+	−	+	−	−	−	−
No. strains (patients*)	10 (10)	10 (7)	3 (3)	12 (12)	4 (2)	2 (1)	2 (1)

* Figure in parenthesis refers to the number of patients from whom the strains were isolated.

they were almost invariably shown to belong to the same biotype; however, in apparently two instances, type 2 and type 4 strains were isolated from the same patient. The only property that these two types differ in is their ability to ferment mannose, but strains that ferment mannose do so only weakly. Perhaps this test should be excluded from a biotyping scheme.

(b) *Serotyping*. Poxton and Byrne (1981b) have shown that four cell-surface antigens can be extracted from *C. difficile* NCTC 11223 with EDTA and visualized by crossed immunoelectrophoresis (CIE) (Fig. 2). Two EDTA antigens (producing precipitin lines 2 and 4) are found in all strains of *C. difficile* so far investigated. Line 4 is often difficult to observe. The antigens that produce lines 1 and 3 may occur together, or singly, or not at all in preparations from some strains. The antigen that produces line 2 is common to *C. sordellii*/*C. bifermentans* and is a membrane associated lipo carbohydrate (Poxton and Cartmill, unpublished work). A fifth antigen, producing line 5, can be extracted from pure cell-walls by NaOH, or by autolysis of whole cells; this is the secondary cell wall polymer (teichoic acid analogue), and it is also common to *C. sordellii*/*C. bifermentans* (Poxton and Cartmill, unpublished). It is possible, therefore, to serotype *C. difficile* by detecting lines 1,

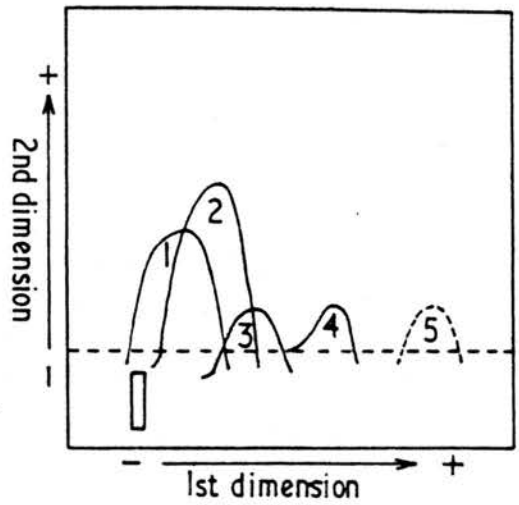

Fig. 2. Diagram of precipitin lines produced by cell surface antigens of *C. difficile* NCTC 11223 in crossed-immunoelectrophoresis. Lines 1–4 are produced by EDTA-extract. Line 5 is produced by the secondary cell wall polymer.

3 and 4 by CIE of EDTA extracts. Isolates from the same patient give the same profile. When sera have been raised against other strains, and when more antigens including the flagella have been investigated, a more complete serotyping system may be developed.

When biotyping and serotyping are used in tandem it should now be possible to investigate the epidemiology of *C. difficile* infections.

5. *New approaches*

The direct visualization of *C. difficile* in clinical specimens would be an obvious advancement in our investigation of *C. difficile* infections. An immunofluorescence assay (IFA) or immunoperoxidase assay would be useful. Hubert *et al.* (1981) have developed an IFA technique for identification of isolated *C. difficile* colonies. They have raised an antiserum against one strain of *C. difficile*. In our studies, antiserum raised against one strain (a different strain from that of the French workers) does not detect all our isolates. We are developing a mixed antiserum to detect all our strains. It is, of course, necessary to absorb *C. difficile* antiserum with *C. sordellii* cells to make it specific.

Problems that we have encountered in IFA applied directly to faeces include the presence of Fc receptors on much of the cellular debris in faeces and the possibility that the bacteria may be antibody-coated.

During the preparation of this paper, a reference has been made to an unpublished finding that many of us have predicted. Bartlett *et al.* (1980) have shown that *C. difficile* produces an enterotoxin as well as a cytotoxin. This enterotoxin appears to be the major virulence factor of the organism. It can be detected in tests with ligated ileal loops in the rabbit or by injection into the caecum of the hamster. It will be necessary to develop simple tests for the detection of the enterotoxin before this can become a routine procedure. The significance of the finding is already clear and it may explain some of the difficulties that we have encountered in relating apparently non-toxigenic strains to disease.

CONCLUSIONS

In so short a period of time, great advances have been made in the isolation and detection of *C. difficile*. This has resulted in an evolving awareness of the range of enteropathies in which it may play a role. Its pathogenic mechanisms are debated, but it is encouraging that the procedures outlined in this short paper promise to make further significant contributions to our understanding and management of conditions in which *C. difficile* may be involved.

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Clostridium difficile in association with sporadic diarrhoea

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Abstract

A total of 154 patients admitted to an infectious diseases unit were included in a year's prospective survey of sporadic diarrhoeal disease. Stools from 19 of them yielded Clostridium difficile, generally on more than one occasion. Twelve of these patients were assessed as having a severe or moderately severe gastrointestinal illness: Cl difficile was the only pathogen isolated from 10 of them, and two had an associated salmonella infection. Seven had had a recent course of antibiotics, but five had not taken antibiotics. Faeces from seven patients with moderate or mild gastrointestinal illness yielded Cl difficile, and two of these patients also had an associated salmonella infection. Two patients in this group had no antibiotic history.

From these findings, the occurrence of C difficile in faeces could not be described as antibiotic-associated. Faecal Cl difficile cytotoxin was detected in only six patients, and generally at low levels. In such patients a more relevant pathogenic index might take account of the numbers of Cl difficile present and of their toxigenic potential.

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Introduction

Though a pathogenic association between *Clostridium difficile* and pseudomembranous colitis is accepted, the role of this organism in intestinal health and disease in infants and adults is still uncertain.[1] The development of better selective culture procedures has greatly facilitated the isolation of *Cl difficile* from stools.[2] We have therefore conducted a prospective study to determine the range of circumstances in which *Cl difficile* may be detected and possibly implicated in sporadic cases of diarrhoea in adults admitted during one year to an infectious diseases hospital.

Patients and methods

We included in the survey all patients admitted to the infectious diseases unit during November 1979 to October 1980 who presented with diarrhoea or developed diarrhoea. During the study period there was no recognised epidemic of gastroenteritis or diarrhoeal disease. Diarrhoea was defined as three or more loose stools daily for one day or more. The stools of patients with diarrhoea were examined for intestinal pathogens, including *Salmonella*, *Shigella*, and *Campylobacter* spp and for *Cl difficile;* a sample of faeces was also referred for virological examination but not for direct electron microscopy. Patients whose stools yielded *Cl difficile* were graded retrospectively according to the severity of their illnesses on clinical grounds alone.

BACTERIOLOGICAL INVESTIGATIONS

Specimens of faeces—Samples were taken with care to avoid contamination and submitted in sterile plastic containers without transport medium. When *Cl difficile* was isolated follow-up specimens were submitted.

Microscopy—Suitable preparations of each specimen were examined by phase-contrast microscopy; a Gram smear was prepared and examined by light microscopy. Pus cells, red blood cells, and Gram-positive rods with spores were noted.

Culture—Approximately 0·1 g or 0·1 ml of faeces was plated on cefoxitin cycloserine fructose agar medium (CCFA)[2] and incubated anaerobically in 90% hydrogen and 10% carbon dioxide for 24 hours at 37°C. Another, similar sample was inoculated into Robertson's cooked meat broth and incubated in the same way. The CCFA plate was examined for typical colonies, and the number of *Cl difficile* in the faeces estimated semi-quantitatively: + = growth in well only (10^2-10^3 organisms/g faeces), + + = growth in well and on primary streaks (10^3-10^4), and + + + = growth in well, primary streaks, and secondary streaks ($\geqslant 10^5$). The cooked meat broth culture was plated on CCFA medium and this plate subsequently examined for *Cl difficile* and scored ¦ if positive only after enrichment. All CCFA plates were incubated for 48 hours before being discarded as negative. All presumptive isolates were confirmed by gas chromatography of the volatile fatty-acid products of metabolism. The acidified supernate from a 24-hour culture in proteose peptone, yeast extract, serum, and glucose medium[3] was examined on a column of 15%

Details of 19 patients from whom Cl difficile was isolated (group 1)

Case No	Age and sex	Severity of illness	Antibiotic or steroid history	Other enteric pathogens isolated	Other illnesses diagnosed	*Cl difficile* isolated	In-vitro toxigenicity titre	Faecal cytotoxin* titre	Treatment and comment
1	42 F	+ + + +				+ +	1 000	64	Vancomycin
2	69 F	+ + + +	Co-trimoxazole†		Rheumatic heart disease	+ + +	100	1 024	Vancomycin. Died
3	55 F	+ + + +			Diabetes, chronic obstructive airways disease	+ +	100	§	Vancomycin
4	77 F	+ + + +			Ischaemic heart disease, chronic obstructive airways disease	+ + +	10 000	§	Vancomycin
5	88 F	+ + + +	Ampicillin + flucloxacillin		Ischaemic heart disease	+ +	1 000	16	Vancomycin, cholestyramine
6	40 M	+ + + +			Cerebral cortical atrophy	+	§	§	Vancomycin
7	24 F	+ + + +	Erythromycin	*Salmonella panama*‡		+ +	10	4	Vancomycin, chloramphenicol
8	65 F	+ + + +	Prednisone, co-trimoxazole,† cefuroxime†	*Salmonella derby*	Rheumatoid arthritis	+ + +	10 000	1 024	
9	87 F	+ + +	Ampicillin		Chronic obstructive airways disease	+	100 000	ND	
10	19 F	+ + +	Penicillin, cephradine, prednisone		Panhypopituitarism	+ + +	10	§	Vancomycin
11	85 F	+ + +			Ischaemic heart disease, hypothyroidism	+	10 000	§	Vancomycin
12	44 F	+ + +	Doxycycline			+	§	§	Cholestyramine (failed), vancomycin
13	50 F	+ +	Ampicillin		Colostomy, diverticulosis	+ + +	100 000	§	Vancomycin
14	84 F	+ +			Secondary myocardial infarction	+	100	§	
15	37 M	+	Oxytetracycline		Hand, foot, and mouth disease	+	100	§	
16	79 M	+	Ampicillin, flucloxacillin†	*Salmonella agona*	Femoral popliteal bypass	+ +	10	16	
17	75 M	+	Chloramphenicol, amoxycillin,† erythromycin	*Salmonella virchow*‡	Chronic obstructive airways disease, pneumothorax	+	§	§	Died from chronic obstructive airways disease
18	19 F	+	Flucloxacillin, lincomycin†		Staphylococcal skin sepsis and eczema	+ + +	§	§	
19	19 I	+				+ +	§	§	

ND = Not done.
‡Organism also isolated from blood cultures.
*Neutralised by *Cl sordellii* antitoxin.
§Not detected in undiluted extract.
†Diarrhoea developed while taking drug

Supelco SP1220, 1% H_3PO_4 on chromosorb W acid washed in a Pye Unicam model 104 gas chromatograph. A typical fatty-acid profile with pronounced peaks of acetic, N-butyric, isobutyric, N-valeric, isovaleric, and isocaproic acids was taken as diagnostic.

Toxin assay—Toxin was assayed essentially according to Bartlett[4] by observing a cytopathic effect on monolayers of human embryonic fibroblast cells. All faecal assays were performed retrospectively on specimens that had been stored at −18°C. Before centrifugation, solid faecal specimens were mixed with a minimum of physiological saline to give a fluid suspension; liquid specimens were used without further dilution. Neutralisation tests were performed with *Cl sordellii* antitoxin (10 μl of a one in 25 dilution added to 90 μl medium over the monolayer just before adding 10 μl of faecal extract). The toxigenicity of fresh isolates of *Cl difficile* was estimated by assaying the supernate from five-day cultures in 3·5% (w/v) brain-heart infusion medium supplemented with 1% (w/v) proteose peptone (both from Oxoid).

Results

A total of 154 patients were included in the survey, 145 of whom were referred by their general practitioners and nine transferred from other hospitals. All were admitted for investigation and treatment of illnesses that either presented with or developed a diarrhoeal phase. Of these, 39 had medical or surgical conditions that might reasonably be associated with diarrhoea and not regarded as primarily infective. Of the remaining 115 patients, 27 (23%) yielded enteropathogenic bacteria, 12 (10%) had salmonella infections, and 15 (13%) yielded campylobacters. A reovirus may have caused another one diarrhoeal episode. From four of our 115 patients a *Salmonella* sp and *Cl difficile* were isolated together. Eighty-seven patients had diarrhoea that might have been infective and was unexplained; from 15 of these we isolated *Cl difficile* alone.

During the survey 35 patients were admitted who did not fulfil the criteria of the study—that is, they did not produce three or more loose stools daily for one day or more. Stools from these patients were also investigated and contained no *Cl difficile*. Since other patients resident in hospital are not entirely equivalent, the 35, like the study patients, were in the main admitted directly from the community, were regarded as matched controls.

Cl difficile was isolated from a total of 19 patients (group 1). Of these, 17 came from their homes and two were from other hospitals. The age range was 19 to 88 years, with a mean of 56; the male to female ratio was 1:4; two patients died; 15 of the patients presented during May to October: and one had travelled abroad recently. There was no case-to-case spread of *Cl difficile*. On only one occasion were two patients with the organism in the ward at the same time. A check on the ward area and clean bedpans during the survey yielded no *Cl difficile*.

Toxin that could be neutralised by *Cl sordellii* antitoxin was detected in the faeces of six patients in group 1. No cytotoxin was detected in any of the specimens that were available for testing (106 tested out of a possible 135) from patients who did not have *Cl difficile* in the faeces (group 2). Twelve of the 19 patients in group 1 and 17 of the 135 in group 2 had had courses of antibiotics during the six weeks before their admission to hospital, whereas only one of the 15 patients

with campylobacter diarrhoea and none of the eight with salmonella infections alone had received antibiotics during that time.

There was no appreciable difference between the two groups in age, sex, mortality, seasonal incidence, contact history, or travel abroad, and the groups were indistinguishable in terms of presentation, biochemical and haematological profiles, and clinical course. Group 2 included a wide variety of acute surgical cases (appendicitis, perforated viscus, obstructed hernia, ischaemic ileitis, and colitis) and medical conditions (diverticular disease, carcinomatosis, pneumonias, ulcerative colitis, and chronic renal failure). There was no obvious correlation with faecal excretion of *Cl difficile* in patients with diarrhoea other than recent antibiotic treatment, which was not an invariable association.

Clinical assessment—The table summarises relevant data for the 19 patients in group 1. The patients were divided retrospectively into four groups based on the severity of their illness; duration, frequency, and persistence of diarrhoea; occurrence of blood in the stools; fever; signs and symptoms of dehydration; increased white cell counts; and low serum albumin concentrations. The four groups were clinically severe (++++), moderately severe (+++), moderate (++), and mild (+). We considered that 12 patients had appreciable enteropathy, and it was among these that the high white cell counts and low serum albumin values were recorded. The symptoms included diarrhoea, vomiting, and abdominal pain; three patients had frank blood in the stools.

Microbiological assessment—Semi-quantitative assessments of the isolation of *Cl difficile* from the 19 patients in group 1 showed many examples of poor correlation with clinical severity. This was also true of our assessments of in-vitro toxigenicity of the *Cl difficile* isolates and it was largely true of our estimations of toxin in the faeces of these patients, though with one exception (case 16) all of those with detectable faecal toxin were severely ill. Five non-cytotoxic strains of *Cl difficile* were isolated; four were associated with diarrhoea in patients from whom no other presumptive pathogen was isolated. Pathogens other than *Cl difficile* were isolated from two severely ill patients (cases 7 and 8). One of these (case 7) had an associated salmonella bacteraemia, and presented with septicaemic shock initially considered to be secondary to an *Escherichia coli* urinary tract infection. With the exception of these two patients, 12 had a clinically severe illness in the absence of any presumptive cause other than *Cl difficile*. Seven of the 12 had received antibiotics within the previous six weeks, but the other five patients had not received antibiotics.

Management—Vancomycin was given to 10 of the 12 patients with clinically severe and moderately severe disease and to one patient with moderate disease. The decision to treat was clinical, though it was influenced by finding *Cl difficile* in the stools. At the time of each patient's illness we did not have the results of the faecal toxin assays; in retrospect it could be argued that several of our patients did not merit specific treatment. Cholestyramine was used only twice; it failed to improve one patient (case 12), who eventually received a course of vancomycin.

Sigmoidoscopy was performed on 11 patients (cases 1-8 and 10-12). Moderate inflammation with contact bleeding was seen in four (cases 1, 3, 7, and 8), but none had pseudomembranes or ulcers; the rest were normal. Rectal biopsy in six patients (cases 1, 3, 5, 6, 7, and 8) showed oedema with chronic or acute inflammatory infiltrates. Barium enemas were performed on two patients, and one showed severe diverticulosis.

Discussion

In a recent survey[5] no *Cl difficile* was isolated from a group of 62 healthy adults, but in an earlier one up to 3% of healthy adults were found to be carrying *Cl difficile*.[6] Most of this work, however, was done before the introduction of the selective CCFA medium.[2] Hence these surveys may have underestimated the carriage rate of *Cl difficile* if some normal adults had carried small numbers of organisms in their stools. Similarly, in our 35 controls we were unable to isolate any *Cl difficile* on CCFA. *Cl difficile* has been isolated from the urogenital tract of men and women,[7] occurs commonly in the faeces of neonates,[5 8] and has an accepted causative association with pseudomembranous colitis in adults. The organism has been associated with 6-48% of cases of antibiotic-associated diarrhoea[9-11]; the assumed association is based on faecal cytotoxin assays or isolations of the organism on selected media. Twelve of our 29 patients with antibiotic-associated diarrhoea yielded *Cl difficile*, but none had pseudomembranous colitis diagnosed. Mogg *et al*[12] and Keighley *et al*[13] have demonstrated the inadequacy of sigmoidoscopy and rectal biopsy and the greater reliability of faecal cytotoxin determinations in diagnosing pseudomembranous colitis.

Evidence is now appearing implicating *Cl difficile* in non-antibiotic-associated colitis,[14-16] exacerbations of chronic inflammatory bowel disease,[17 18] and postoperative diarrhoea.[10 13] The clustering of some cases suggests that cross-infection may occur.[19]

Falsen *et al*[11] found that *Cl difficile* was the second commonest enteropathogenic isolate in a survey of many stool specimens submitted to a laboratory. Our hospital-based survey would have excluded many young patients with salmonella and campylobacter infections treated by general practitioners without referral to hospital. In our series *Cl difficile* was the commonest presumptive enteric pathogen. Unknown or undetected pathogens might have accounted for some of our cases of presumed infective diarrhoea. For example, the occurrence of rotaviruses and other enteropathogenic viruses in an adult population has not been adequately assessed and merits further consideration. With these provisos, *Cl difficile* seems to be associated with some cases of diarrhoea requiring admission to hospital and may be acting as a primary pathogen in a proportion of these. In common with the findings of others,[11 20] we were able to isolate an accepted infective agent from only 28 (24%) of 115 patients. Our suggestion that *Cl difficile* may be an additional accepted cause of infective diarrhoea is supported by recent reports of hospital studies[19 21] and animal studies.[22 23]

The titres of faecal cytotoxin detected in our survey (from 0-1024) were appreciably lower than those obtained in classic cases of pseudomembranous colitis (500-400 000[24], 1000-5000,[25] and 1000-2000[26]). Our patients may have had illnesses at the

lower end of the range of clinical severity. The *Cl difficile* isolates from five of our patients were non-cytotoxigenic, which raises the question whether non-cytotoxigenic *Cl difficile* is invariably non-pathogenic. Recent work suggests that the cytotoxin presently assayed is not the enterotoxin that causes the diarrhoea.[27]

When *Cl difficile* was isolated, a decision to treat was made on clinical grounds with oral vancomycin[28] or with cholestyramine.[29] That non-cytotoxigenic strains of *Cl difficile* might be implicated in diarrhoea led us to regard vancomycin as the first choice when specific treatment seemed to be indicated. The criteria required to implicate *Cl difficile* as an enteric pathogen are not yet clear. It may be helpful to avoid the terms toxigenic and non-toxigenic until the role of the cytotoxin and enterotoxin in relation to enterotoxicity is defined. It is important to determine whether a relation might exist between the numbers of organisms excreted, their ability to produce one or more toxic factors, the concentrations of these factors in the faeces, and the clinical condition of the patient. The epidemiology is complex,[30] and the enteropathogenic range of *Cl difficile* is as yet undefined. We suggest that the range of illness produced by *Cl difficile* may include sporadic infective diarrhoea and that oral vancomycin should be considered for patients whose clinical condition causes anxiety.

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Analysis of the membrane lipocarbohydrate antigen of *Clostridium difficile* by polyacrylamide gel electrophoresis and immunoblotting

(*Clostridium difficile*, lipoteichoic acid, antigen, immunoblotting, silver staining)

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1. SUMMARY

The membrane lipocarbohydrate antigen (lipoteichoic acid analogue) of *Clostridium difficile* has been purified by aqueous phenol extraction and Sepharose 6B chromatography. After analysis by polyacrylamide gel electrophoresis (PAGE) and immunoblotting it has been shown to consist of a series of components of differing M_r. It appears as a regularly spaced ladder pattern similar to those shown for the lipopolysaccharide (LPS) of many Gram-negative bacteria.

2. INTRODUCTION

C. difficile is a Gram-positive, spore-forming, anaerobic bacterium and is well recognised as being the major cause of pseudomembranous and antibiotic-associated colitis [1]. The structure of the cell-surface appears to be typically Gram-positive: components analogous to wall and membrane teichoic acids have been demonstrated [2] and a regular surface array of protein subunits has been recognised [3]. The membrane teichoic acid analogue is not based on a poly-glycerol phosphate backbone as in most other Gram-positive bacteria so far investigated, but contains glucose, glucosamine, phosphate and fatty acid in the molar proportions of 2 : 1 : 1.6 : 0.04 [2].

In Gram-negative bacteria the LPS has been investigated by PAGE. After silver staining, it has been shown to be made up of a heterogeneous population of differing M_r, adjacent bands varying by one repeating unit of polysaccharide [4].

It might be expected that the appearance on PAGE of lipoteichoic acid molecules based on the typical poly-glycerol phosphate backbone would not show the characteristic ladder pattern of LPS. If the chain lengths were variable they would only differ by a single substituted glycerol phosphate unit, which might not be resolved on PAGE.

This present study uses PAGE and immunoblotting to investigate the immunochemistry of the atypical lipocarbohydrate of *C. difficile*. Antigenically related species are also compared.

3. MATERIALS AND METHODS

3.1. Culture of organisms and cell breakage

The strains used were *C. difficile* NCTC11223, and 3 clinical isolates, RIE11831, MPRL161 and MPRL597, *C. sordellii* NCTC8780 and *C. bifermentans* NCTC506.

A 0.1% (v/v) inoculum of an overnight culture

of bacteria in Robertsons' cooked meat broth was added to 6 l of pre-reduced proteose peptone yeast extract medium containing 0.04% (w/v) sodium carbonate and 0.075% (w/v) cysteine hydrochloride [5] and incubated anaerobically at 37°C for 18 h in an anaerobic cabinet (Forma Scientific). The bacteria were harvested (8500 × *g* for 10 min at 4°C) and broken as described previously [2]. After removal of the cell walls at 45 000 × *g* for 20 min, the supernate, containing the membrane antigen, was lyophilized prior to further extraction.

3.2. Extraction of membrane antigen

The lyophilized supernate was defatted with 2 × 200 ml chloroform/methanol (2 : 1, v/v) over 24 h and extracted with cold 80% (w/w) phenol as described by Coley et al. [6]. The membrane material (about 0.8 g) was suspended in distilled water to about 10% (w/v), mixed with an equal volume of 80% aqueous phenol (w/w) and stirred at room temperature for 30 min. This was centrifuged at 2500 × *g* for 20 min at 4°C. The upper aqueous layer, after dialysis, was mixed with an equal volume to 0.2 M acetic acid/acetate buffer, pH 5.0, containing 0.02 M $MgCl_2$. Ribonuclease and deoxyribonuclease (Sigma) were added and the mixture incubated under toluene at 37°C overnight. Phenol/water extraction was repeated, the upper aqueous layer dialysed and finally lyophilized. This was the crude membrane antigen.

Further purification of the MPRL161 sample (13 mg in 500 μl of distilled water) was carried out by fractionating on a Sepharose 6B column (30 × 1 cm) by the method of Coley et al. [6].

3.3. Immunoelectrophoresis

Crossed immunoelectrophoresis (CIE) was performed by the procedure of Weeke [7] as described by Poxton and Byrne [8] with antiserum raised to MPRL161. Samples (10 μl) were applied at a concentration of 10 mg/ml in distilled water.

Fused rocket immunoelectrophoresis (FRIE) was done with antiserum raised to NCTC11223 as described by Svendsen [9] with the same buffer system as used for CIE. The pooled fractions were desalted on a 10 ml Sephadex G25 column.

3.4. Preparation of antiserum

Antisera against UV-killed, whole, washed cells of *C. difficile* NCTC11223 and MPRL161 were prepared as described previously [8].

3.5. PAGE and immunoblotting

The buffer system of Laemmli [10] with 10% slab gels as described by Poxton and Brown [11] with and without SDS was used. Samples (50 μl) containing 250 μg crude antigen or 25 μg purified antigen were run on each track. Gels were stained with silver [4] for 10–15 min. For immunoblotting gels without SDS were used. Materials were transferred to nitrocellulose membranes (0.2 μm pore, Sartorius) and treated with antiserum raised against NCTC11223 and anti-rabbit HRP conjugate, as previously described [12], except that Tween 20 was omitted from the washing solutions.

4. RESULTS

Crude aqueous phenol-extracted membrane antigens from 4 strains of *C. difficile* and one strain each of *C. sordellii* and *C. bifermentans* were analysed by SDS–PAGE and stained with silver. The results (Fig. 1) showed that the patterns produced by the *C. difficile* strains all appeared as a regularly spaced ladder pattern reminiscent of the pattern produced by the LPS of Gram-negative bacteria. The patterns produced by the other two species were indistinct, and even when more material was applied to the gel no ladder pattern could be demonstrated. Immunoblots of the *C. difficile* extracts with antiserum raised to *C. difficile* MPRL11223 revealed patterns of faint antigenic bands similar to those of the silver stain (results not shown).

One of the crude antigens (from *C. difficile* MPRL161) was analysed by CIE and produced a pattern identical to that previously produced by NCTC11223, i.e., two precipitin arcs showing partial identity [2]. After fractionation of the MPRL161 crude phenol extract on a Sepharose 6B column, two antigens were detected with FRIE, one eluting in the void volume, the other with a K_{av} of 0.71. This was similar to that previously shown for NCTC11223 [2]. The fractions corresponding to the two antigens were pooled, desalted and analysed further by PAGE and im-

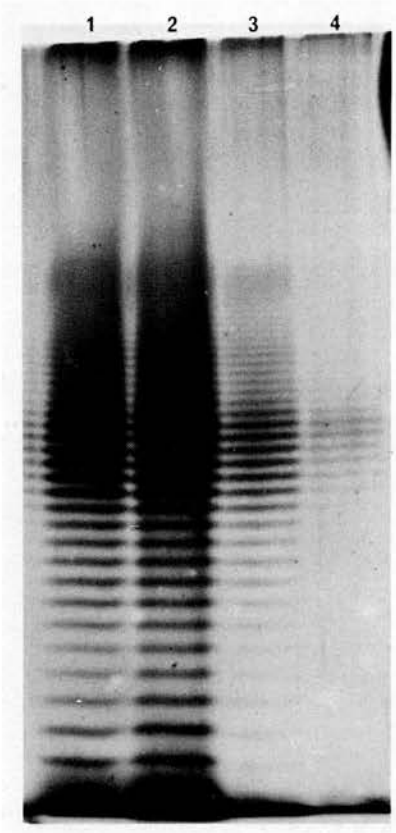

Fig. 1. Silver-stained SDS–PAGE of crude aqueous-phenol extracts of *C. difficile*. Lane 1, NCTC11223; lane 2, MPRL161; lane 3, RIE118311; lane 4, MPRL597. Samples (50 μl) containing 250 μg crude antigen were applied to each lane.

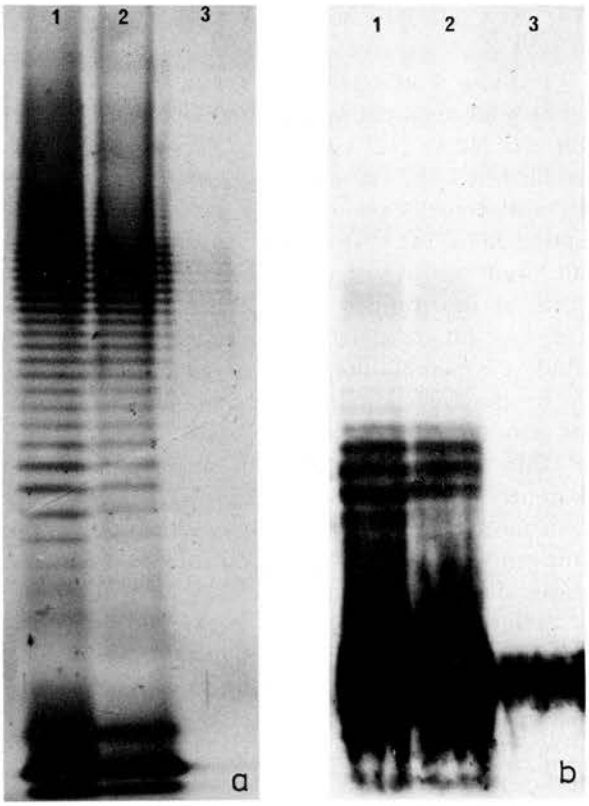

Fig. 2. (a) Silver-stained PAGE (without SDS) of crude aqueous-phenol extract of MPRL161 (lane 1) and the two antigenic fractions detected by FRIE from the Sepharose 6B column (lane 2 is first antigen and lane 3 the second). Lane 1 contained 250 μg crude antigen, and lanes 2 and 3 25 μg purified antigen. (b) Corresponding immunoblot with antiserum raised against NCTC11223.

munoblotting. Initial attempts to obtain good photographs of immunoblots were hampered by the apparent lack of sensitivity of the procedure. Subsequently, sodium dodecyl sulphate (SDS) was omitted from the gels but retained in the sample and electrode buffers. This resulted in greater sensitivity of detection by immunoblotting, and the silver-stained gel was extremely similar to the SDS-containing gel, except that the bands ran slower when SDS was omitted. The results of the PAGE and corresponding immunoblot of the crude membrane antigen and the two antigenic fractions after Sepharose 6B treatment are shown in Fig. 2. The material in fraction 1 gave an identical banding-pattern to that seen with the crude material. In the silver stain there was nothing apparent in the sample corresponding to fraction 2. In the immunoblot of fraction 2 an antigenic band was detected just behind the gel front.

Immunoblotting with antiserum to NCTC11223 of the crude extracts of the four *C. difficile* strains and the other two species was repeated, omitting SDS. The patterns produced by *C. difficile* were identical to those obtained with the silver stain. There was distinct cross-reaction of the *C. difficile* antiserum with the extracts from *Clostridium sordellii* and *Clostridium bifermentans*, part of the pattern in the mid-region of the *C. sordellii* and *C. bifermentans* gels being series of fine lines which appeared as a smear in the silver stain (not shown).

5. DISCUSSION

Previous work has shown that a phenol extract of lyophilized supernate from broken cells of *C. difficile* NCTC11223 contained two antigens when studied by CIE. These antigens, which appeared to be antigenically related, were proposed to correspond to (a) the lipoteichoic acid moiety found in all Gram positive organisms and (b) a deacylated form of this antigen which cross-reacts with the cell wall antigen (teichoic acid) [2]. In this present study the membrane carbohydrate has been shown to be extremely similar in appearance to the LPS of Gram-negative bacteria when examined by PAGE, giving a ladder pattern; each rung apparently differing by one repeating unit of polysaccharide. Relatively high concentrations of the antigen were applied to the gels to ensure that the silver-stained material developed rapidly, thus avoiding problems with high background staining. The identity of the second antigenic peak from the Sepharose 6B column is uncertain. This rather heterogeneous low-M_r material may be fragmented wall antigen or deacylated membrane antigen.

As far as we are aware this is the first report of the analysis of a lipoteichoic acid (or analogue) by PAGE. It has however been speculated that a ladder pattern seen in immunoblots of crude cell surface extracts of *Clostridium botulinum* might be due to such a molecule [13]. We do not know if this ladder pattern is produced by the LTA analogues of other clostridia or indeed of other Gram-positive bacteria.

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The cell wall proteins of *Clostridium difficile*

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1. SUMMARY

The proteins which can be released by 6 M urea treatment from the cell walls of *Clostridium difficile* represent the major cell surface proteins. In the 5 strains examined there are one to three of these major proteins. They appear to be strain-specific antigens being detected in immunoblots only with homologous antiserum. A common cell-surface protein of M_r 73 kDa has been identified as a minor component of the urea extract.

2. INTRODUCTION

Clostridium difficile is the anaerobic, spore-forming organism responsible for most, if not all cases of antibiotic-associated pseudomembranous colitis [1]. Molecules analogous to the teichoic acids and lipoteichoic acids found in typical Gram-positive organisms have been identified in the cell envelope [2,3]. An outer cell wall layer has also been identified consisting of two major protein molecules organised in a regular array [4]. By SDS-PAGE all isolates examined could be divided into two different groups based on the molecular mass (M_r) values of these major wall proteins which had been extracted with urea [4].

In the development of a fingerprinting system for epidemiological investigations of outbreaks of *C. difficile*-associated infection [5,6] we have observed an apparently infinite number of EDTA extractable cell-surface protein profiles. Tabaqchali and coworkers [7,8] have developed a typing scheme based on radiolabelling of major cell proteins and the patterns produced have allowed recognition of at least 9 types. Immunoblotting studies with whole cell antiserum showed that the major radiolabelled proteins were antigenic when probed with homologous antiserum. They did not cross-react with heterologous antiserum and might correspond to major cell surface proteins [8]. However, by both radiolabelling and immunoblotting there was an indication that all strains could be divided into many more groups than the two originally defined by SDS-PAGE of the urea-extractable regular array proteins [4].

The aim of this study was to investigate the urea-extractable cell wall-associated proteins by SDS-PAGE and immunoblotting and relate them to the fingerprinting/typing schemes.

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3. MATERIALS AND METHODS

3.1. Strains and culture conditions

Five strains of *C. difficile*, which from previous work were known to produce very different surface protein fingerprints, were selected for this study: NCTC 11223, MPRL 604, MPRL 683, MPRL 1123 and MPRL 1128 (MPRL numbers refer to our departmental culture collection). Organisms were cultured anaerobically for 17 to 18 h at 37 °C in pre-reduced proteose peptone, yeast extract medium (PPY) containing 0.04% (w/v) sodium carbonate and 0.075% cysteine hydrochloride [9].

3.2. Preparation of EDTA extracts

These were prepared from 100 ml of bacterial culture in PPY medium as described previously [6].

3.3. Cell wall preparation and urea extraction of proteins

Cells were cultured in 10 litres of PPY medium, harvested and washed once (10 000 × *g*, 10 min, 4 °C). The wash buffer consisted of 0.05 M Tris/HCl, 0.025 M $MgCl_2$ and 0.85% NaCl, pH 7.4. All steps were done on ice. Cells were resuspended in 40 ml of buffer, DNase and RNase (Sigma—both proteinase free) were added and the cells disrupted by French Pressing as previously described [2]. After centrifugation at 20 000 × *g* (30 min, 4 °C) the resulting pellet was washed four times in ice-cold distilled water (centrifuging 30 000 × *g*, 15 min). The pellet was resuspended in 10 ml of distilled water and stored at −20 °C.

The crude cell wall preparation was purified and extracted with urea as described in [4]. The cell wall suspension (1 ml) was separated into pellet and supernate by centrifugation (50 000 × *g*, 10 min, 4 °C) and the pellet resuspended in 1 ml of 2% (v/v) Triton-X100 in distilled water. This was left at room temperature for 30 min. Following two washes in distilled water it was resuspended in 1 ml of 6 M urea and again kept at room temperature for 30 min. Following separation into pellet and supernate (50 000 × *g*, 10 min, 4 °C) the supernate was retained and stored at −20 °C.

3.3. SDS-PAGE and immunoblotting

The buffer system of Laemmli [10] with 10% slab gels was used. Samples (50 μl) containing 25 μg of protein (determined by the method of Lowry [11] with bovine serum albumin as standard) were run in each track. Gels were stained with Coomassie blue. For immunoblotting, extracts were transferred to nitrocellulose membranes (0.2 μm pore, Sartorius), probed with both absorbed and unabsorbed whole cell antiserum raised in rabbits (see below) and visualised with anti-rabbit HRP conjugate as previously described [5].

3.4. Preparation and absorption of antisera

Antiserum was raised in rabbits by intravenous injection of U.V.-killed whole cells, without adjuvant, of the five strains listed above (3.1.) by the method of Poxton and Byrne [12].

Absorption was as follows: bacteria harvested from 50 ml of culture were washed twice in phosphate buffered saline (50 mM phosphate buffer, pH 7.4 containing 0.15 M NaCl). Each antiserum was absorbed with the four heterologous bacteria at the same time by mixing the pooled pellets with 1 ml of antiserum. The suspensions were mixed for 30 min at 37 °C on a blood-cell suspension mixer. Bacteria were removed by centrifugation at 5 000 × *g* for 30 min at room temperature. This absorption procedure was repeated once.

4. RESULTS

From previous epidemiological studies it was known that the five strains selected each produced a different profile when whole-cell EDTA extracts were examined on SDS-PAGE. When the urea extracts of the walls of these five strains were examined by SDS-PAGE the patterns produced were again all different (Fig. 1). All strains produced between 1 and 3 major protein bands all differing from each other in M_r. Several other bands of weaker intensity were also present in the

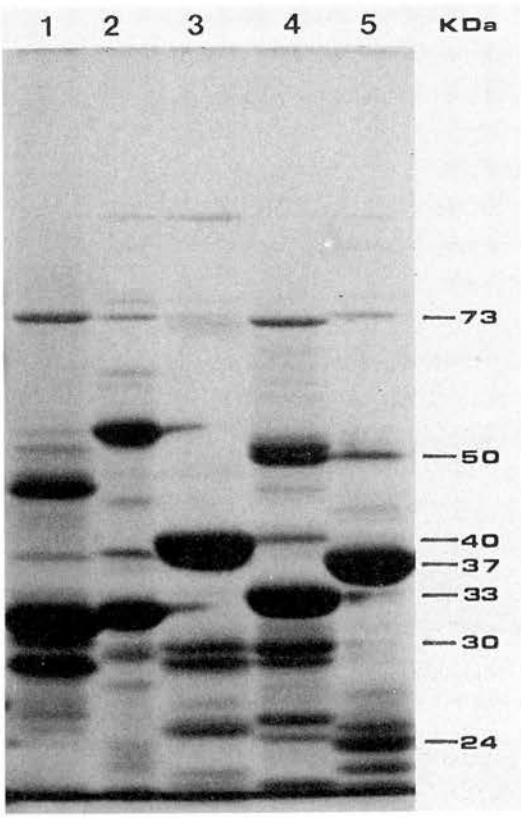

Fig. 1. Coomassie blue stained SDS-PAGE (10% gel) of the proteins (25 μg) extracted from the cell walls of five strains of *C. difficile* with 6 M urea. Track 1, NCTC 11223; track 2, MPRL 604; track 3, MPRL 683; track 4, MPRL 1123; track 5, MPRL 1128.

urea extracts. In general the major bands in the urea extract corresponded to the major bands of the more complex EDTA extracts (Fig. 2). The exception being strain NCTC 11223 where only one of the three major urea extracted bands was a major component of the EDTA extract.

When the urea extracts were electrophoretically transferred to nitrocellulose and probed with homologous antiserum, the major protein bands were revealed as the major antigens. However, a band of M_r 73 kDa which stained only weakly with Coomassie blue was also revealed as a major antigen and it was present in all of the strains. On probing with heterologous antisera the major urea-extracted proteins were not cross-reactive, but the apparently common major antigen at 73 kDa was detected. Also many of the minor antigens cross-reacted. Examples of the five strains probed with antisera raised against MPRL 604 and MPRL 1123 are shown in Figs. 3a and 4a respectively. The reactions between MPRL 1128 antigen and the MPRL 1123 antiserum was complicated by the strong cross-reaction of the antigen at 37 kDa (Fig. 4a, track 5) which was not apparent in the Coomassie blue stained gel of MPRL 1123 (Fig. 1, track 4). When the homologous immunoblot is examined (Fig. 4a, track 4) there is, however, a corresponding antigen in the MPRL 1123 track.

When homologous serum that had been absorbed with the four other heterologous strains was used (Figs. 3b and 4b), the patterns became much simpler because of the removal of the cross-reactions. The major antigens were generally strain-specific and retained their prominence. The exception was the common 73 kDa antigen. In most tracks there was no obvious reduction in its staining intensity as might have been expected if it was common. On close examination this band of approximately 73 kDa varies slightly in M_r and in strain MPRL 683 (track 3 in Figs. 3a and 4a) it is made up of two closely spaced bands.

5. DISCUSSION

From the work presented here it is apparent that the major proteins of the surface of *C. difficile* are highly variable in their M_r. This disagrees with the previously published work where it was reported that only two SDS-PAGE patterns could be produced by the proteins which are extracted by urea and are responsible for the regular arrays found on the surface of *C. difficile* [4]. With the exception of the series of major antigens of approximately 73 kDa, the major proteins extracted by urea are strain specific antigens. This might form the basis of the typing scheme developed by Tabaqchali and coworkers [7] where the patterns of ^{35}S methionine incorporation reflect the major cell surface protein patterns. In our own fingerprinting method [6] we have observed an apparent infinite number of patterns of surface proteins by SDS-PAGE. By extending our examination of the fingerprinting by immunoblot-

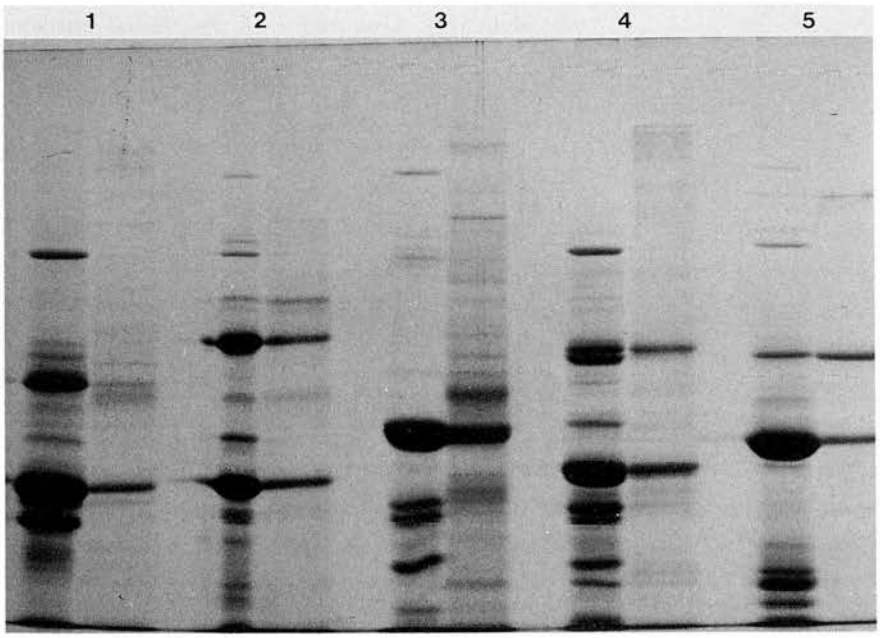

Fig. 2. Coomassie blue stained SDS-PAGE comparing the urea-extracted cell-wall proteins (left-hand track) with the proteins extracted by EDTA from whole cells (right-hand track). Lanes 1–5 are as in Fig. 1.

ting we can, with the use of one carefully chosen antiserum (raised to whole cells of NCTC 11223), detect differences between strains by observing the fingerprint which results from detecting the minor surface antigens. This appears to result in a simple, possibly unique fingerprint for each strain of *C. difficile*.

That the common (73 kDa) antigen was not removed by absorption might have been for several reasons: between strains it varies slightly in M_r

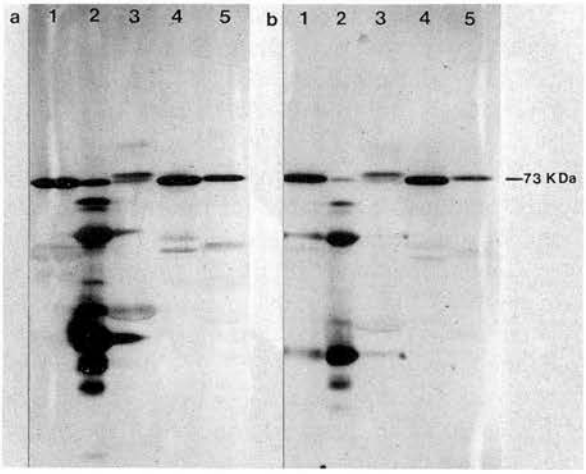

Fig. 3. Immunoblots of SDS-PAGE of the five strains of *C. difficile* as in Fig. 1 probed with (a) unabsorbed antiserum raised to MPRL 604 and (b) the antiserum after absorption with whole cells of the four heterologous strains. The homologous reaction is in track 2.

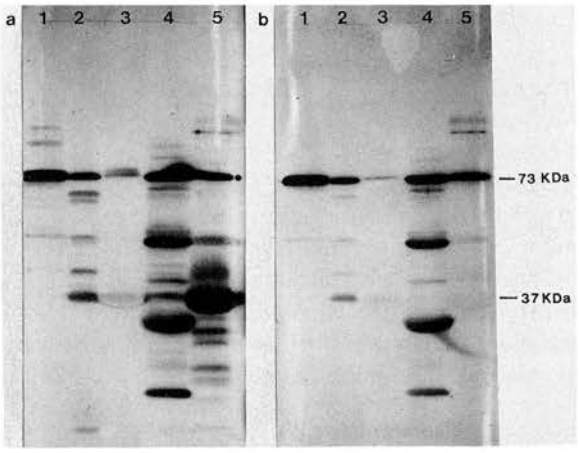

Fig. 4. Immunoblots of SDS-PAGE of the five strains of *C. difficile* as in Fig. 1 probed with (a) unabsorbed antiserum raised to MPRL 1123 and (b) the antiserum after absorption with whole cells of the four heterologous strains. The homologous reaction is in track 4.

but it may have sufficiently different epitopes to appear distinct, or, perhaps more likely, it is because it cannot absorb the antibody because it is present in such small amounts or it is masked at the cell surface of the whole bacteria.

From the work of Tabaqchali and coworkers [8] and of Delmée and coworkers [13] it appears that only certain of their types of *C. difficile* are commonly associated with disease, while other types are more associated with asymptomatic carriage. If, as appears likely, our cell wall-associated protein patterns correlate with the ^{35}S patterns of Tabaqchali [7], and if the serotyping system of Delmee [13] is due to cell-surface protein antigens, it may be that these proteins represent as yet undefined virulence factors of the organism.

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IMMUNOCHEMICAL FINGERPRINTING OF *CLOSTRIDIUM DIFFICILE* STRAINS ISOLATED FROM AN OUTBREAK OF ANTIBIOTIC-ASSOCIATED COLITIS AND DIARRHOEA

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SUMMARY. Twenty eight strains of *Clostridium difficile*, isolated from an outbreak of antibiotic-associated colitis and diarrhoea in an orthopaedic ward and from sporadic cases throughout Sweden, were sent to Edinburgh for immunochemical fingerprinting without information about their origin. EDTA extracts of the organisms were examined by crossed immunoelectrophoresis (CIE), polyacrylamide gel electrophoresis (PAGE) and electroblot transfer. Two patterns were revealed by CIE: group A (18 strains) and group B (10 strains). PAGE and electroblot transfer revealed one major group of 10 strains (group 1), six small groups of two or three strains and six strains which were unlike any other strain. The CIE group B and PAGE-electroblot group 1 were identical. Nine of the 10 strains in this group were from patients in the outbreak. These findings indicate that a single strain spread in the orthopaedic ward as a nosocomial infection and that this strain differed from most other strains investigated. The PAGE-electroblot technique should, therefore, greatly aid investigations into the epidemiology of *C. difficile* infections.

INTRODUCTION

Clostridium difficile is now well recognised as the major cause of antibiotic-associated pseudomembranous colitis (Larson *et al.*, 1978; Bartlett, 1979; Möllby, Nord and Aronsson, 1980) and appears also to be responsible in part for a spectrum of related bowel conditions, such as antibiotic-associated diarrhoea, post-operative diarrhoea, exacerbations of chronic inflammatory bowel disease and sporadic non-antibiotic-associated diarrhoea (British Medical Journal, 1981; Brettle *et al.*, 1982). Carriage of *C. difficile* by healthy adults is extremely uncommon but high isolation rates of up to 40% have been reported for infants (George, Sutter and Finegold, 1977).

The epidemiology of *C. difficile* is still poorly understood. Animal studies (Larson, Price and Borriello, 1980) and the clustering of cases (Howard, Sullivan and Troster,

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1980; Greenfield *et al.*, 1981; Rogers *et al.*, 1981) suggest that the organism may be transmitted from person to person or from the environment (Mulligan *et al.*, 1980; Kim *et al.*, 1981) rather than being a simple overgrowth of a minor component of the normal flora in a compromised bowel. Even healthy carriage by infants appears to be associated with case-clustering (Larson *et al.*, 1982). More thorough investigations into the modes of transmission of *C. difficile* have been hampered by the lack of a reliable typing system for this species. The aim of the present study was to show by the use of immunochemical fingerprinting that isolates of *C. difficile* obtained from a nosocomial outbreak of antibiotic-associated colitis and antibiotic-associated diarrhoea in an orthopaedic ward were identical. These isolates were also different from 15 isolates from other sources. Crossed immunoelectrophoresis, SDS-polyacrylamide gel electrophoresis and electroblot transfer were used to identify the various strains of *C. difficile*.

Materials and Methods

Patients. During a period of 14 days, stool samples from patients on antibiotic therapy in two different wards were analysed for the presence of *C. difficile* cytotoxin and organisms. In an orthopaedic ward at Huddinge Hospital, Stockholm, where there was an outbreak of *C. difficile*-associated colitis and diarrhoea during this period, 34 patients (20 females, 14 males; mean age 74 years) were investigated, and during the same period 29 patients (17 females, 12 males; mean age 59 years) in the Department of Infectious Diseases, Karolinska Institute, Roslagstulls Hospital, Stockholm, with only sporadic incidents of this disease, were investigated. Patients were regarded as having antibiotic-associated colitis (AAC) on the basis of endoscopic examination, or as having antibiotic-associated diarrhoea (AAD) if they had more than five loose stools daily and endoscopy was negative or, as was the case for the majority of these cases, if endoscopy was not performed. The antimicrobial agents used in the two wards were clindamycin, cephalosporins and β-lactamase-stable penicillins, and the patterns of use were roughly comparable between the wards.

Bacterial isolates. Isolation and identification of *C. difficile* and testing for toxin production were done as previously reported (Aronsson, Möllby and Nord, 1981). Fifteen isolates of *C. difficile* from 13 patients in the orthopaedic and infectious diseases wards were fingerprinted immunochemically along with two isolates from another two patients in the orthopaedic ward, one sampled 4 months before and the other 2 months after the outbreak in the orthopaedic ward. Another 11 isolates from 10 patients with AAC or AAD in four other hospital wards in Sweden were also included. All strains were toxigenic and they were stored as cooked-meat-broth cultures. They were sent to Edinburgh for fingerprinting without information about their origin.

Environmental sampling. Stool samples from 37 members of the staff at the orthopaedic ward at Huddinge Hospital were collected and assayed for *C. difficile* and its toxin. Plastic Rodac plates (Falcon, Oxnard, CA) were used to culture environmental samples from beds, toilets, washing rooms, treatment rooms and floors.

Culture of organisms and antigen preparation. Stationary-phase cooked-meat-broth cultures (0·1 ml) were inoculated into 100-ml volumes of PPY medium (Holbrook, Duerden and Deacon, 1977), which contained sodium carbonate 0·04% w/v and cysteine hydrochloride 0·075% w/v, and incubated anaerobically with CO_2 10% for 16 h at 37°C. Spore formation was negligible as judged by phase-contrast microscopy. Antigens were extracted with EDTA by a method simplified from that of Poxton and Byrne (1981). Bacteria were harvested by centrifugation (20 000*g* for 8 min), washed twice in phosphate-buffered saline (PBS; 50 mM sodium phosphate buffer, *p*H 7·4, containing 0·15 M NaCl). The drained pellet was resuspended in 1 ml of PBS containing 10 mM EDTA and incubated for 30 min at 45°C. The extracted cells were removed by centrifugation (20 000 *g* for 10 min) and the supernatant fluid was used undialysed as antigen. Protein content of the preparation was measured by the method of Lowry *et al.* (1951).

Antiserum preparation. Antisera against u.v.-killed cells of *C. difficile* NCTC11223 and two clinical isolates not from the present study were raised in New Zealand White rabbits by the method of Poxton and Byrne (1981).

Crossed immunoelectrophoresis. This was performed by the procedure of Weeke (1973) as described by Poxton and Byrne (1981). EDTA antigens were prepared in duplicate from all the strains. Protein concentrations in the preparations were *c.* 1–3 mg/ml.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The buffer system of Laemmli (1970) was used with 10% slab gels and the method was that of Poxton and Brown (1979). A 20-track gel was used to run two sets of nine EDTA antigens at the same time. One set was stained with Coomassie Blue and the other was used for transfer to a nitrocellulose membrane.

Electroblot transfer. The method of Towbin, Staehelin and Gordon (1979) was followed. Briefly, the EDTA extract that had been separated on SDS-PAGE was transferred to nitrocellulose membrane (Transblot ™ Transfer Medium, BioRad) in a Tris, glycine, methanol buffer, *p*H 8·3 (Towbin *et al.*, 1979) at 12 V and 40 mA for 18 h. The antigens were probed with the antiserum raised against *C. difficile* NCTC11223 by the Immun-blot™ immunoassay (BioRad): after washing for 10 min in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl, *p*H 7·5), the unbound sites on the membrane were blocked by treatment with gelatin 3% in TBS for 45 min. The membrane was transferred to the *C. difficile* antiserum diluted 1 in 200 in gelatin 1% in TBS and incubated at room temperature for 3 h. After two 10-min washes in Tween 20 0·025% in TBS, the membrane was placed into goat anti-rabbit IgG-horseradish peroxidase conjugate (BioRad) diluted 1 in 3000 in gelatin 1% in TBS and incubated at room temperature for 1 h. After two more washes as above, the membrane was placed into HRP colour development solution (BioRad) which contains 4-chloro-1-naphthol. The colour developed between 5 and 15 min. Several changes of distilled water were used to stop the reaction. All of the above steps were performed with gentle agitation.

RESULTS

In the infectious diseases ward with only sporadic cases of AAC or AAD, no patient on antibiotic treatment developed serious diarrhoea during the time of investigation, and only two of 29 patients were found to be colonised. In the orthopaedic ward, six (four females, two males; mean age 75 years) out of 34 patients developed serious colitis that needed vancomycin therapy. Another seven patients (five females, two males; mean age 72 years) colonised with *C. difficile* showed no or minor intestinal symptoms (table I). In total, 15 strains from these two wards were submitted for immunochemical fingerprinting without information about their origins.

Initially, a sample of each antigen was run in CIE against three different antisera—one raised against the reference strain *C. difficile* NCTC11223, the others

TABLE I

Acquisition of C. difficile by patients on antibiotic therapy in two different wards

Ward	Number of patients	Number of patients (%) who developed colitis	Number of patients (%) who were colonised but asymptomatic
Orthopaedic ward with *C. difficile* outbreak	34	6 (18)	7 (20)
Infectious diseases ward with sporadic cases	29	0 (0)	2 (7)

TABLE II

Groups of C. difficile identified by CIE and SDS-PAGE-electroblot analysis

CIE group	SDS-PAGE-electroblot group	Number of strains	Source
B	1	9*	O.W.
B	1	1	S
A	2	3(2)†	S
A	3	2	S
A	4	3	S
A	5	2(1)†	O.W.
A	6	2	S
A	–	2(1)†	O.W.
A	–	2	I.D.
A	–	2	S

O.W. = orthopaedic ward; I.D. = infectious disease ward; S = from hospital wards throughout Sweden.

* Four patients with AAC and five carriers.

† Two strains were isolated from the same patient.

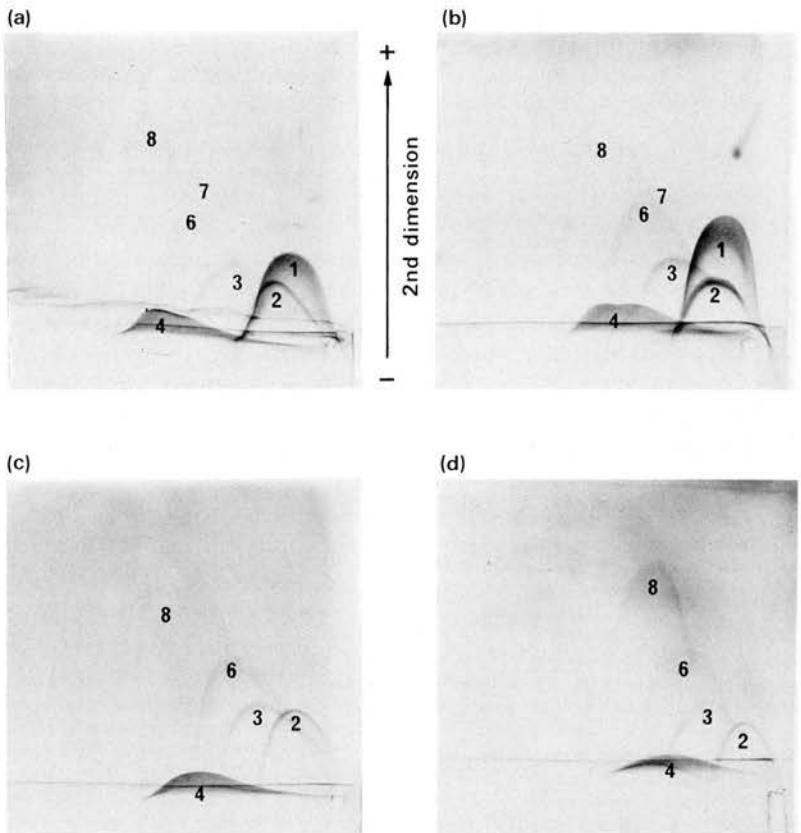

FIG. 1.—Examples of CIE patterns of EDTA extracts of *C. difficile* run against antiserum to whole cells of *C. difficile* NCTC11223. Approximately 15 μg of protein in 10 μl in the first dimension was run into 250 μl of antiserum in the second dimension; (a) and (b) are examples of group-B patterns and (c) and (d) are examples of group-A patterns.

against two clinical isolates. Only the patterns of precipitin lines produced by the NCTC11223 antiserum were considered suitable for further investigation. The patterns produced by the antisera to the clinical isolates were ill-defined. Only line 4 (see below) was at all clear; they were difficult to compare and were not used further.

After all the antigens had been tested with the reference antiserum, it was seen that the duplicate antigen preparations gave identical patterns, but that two distinct

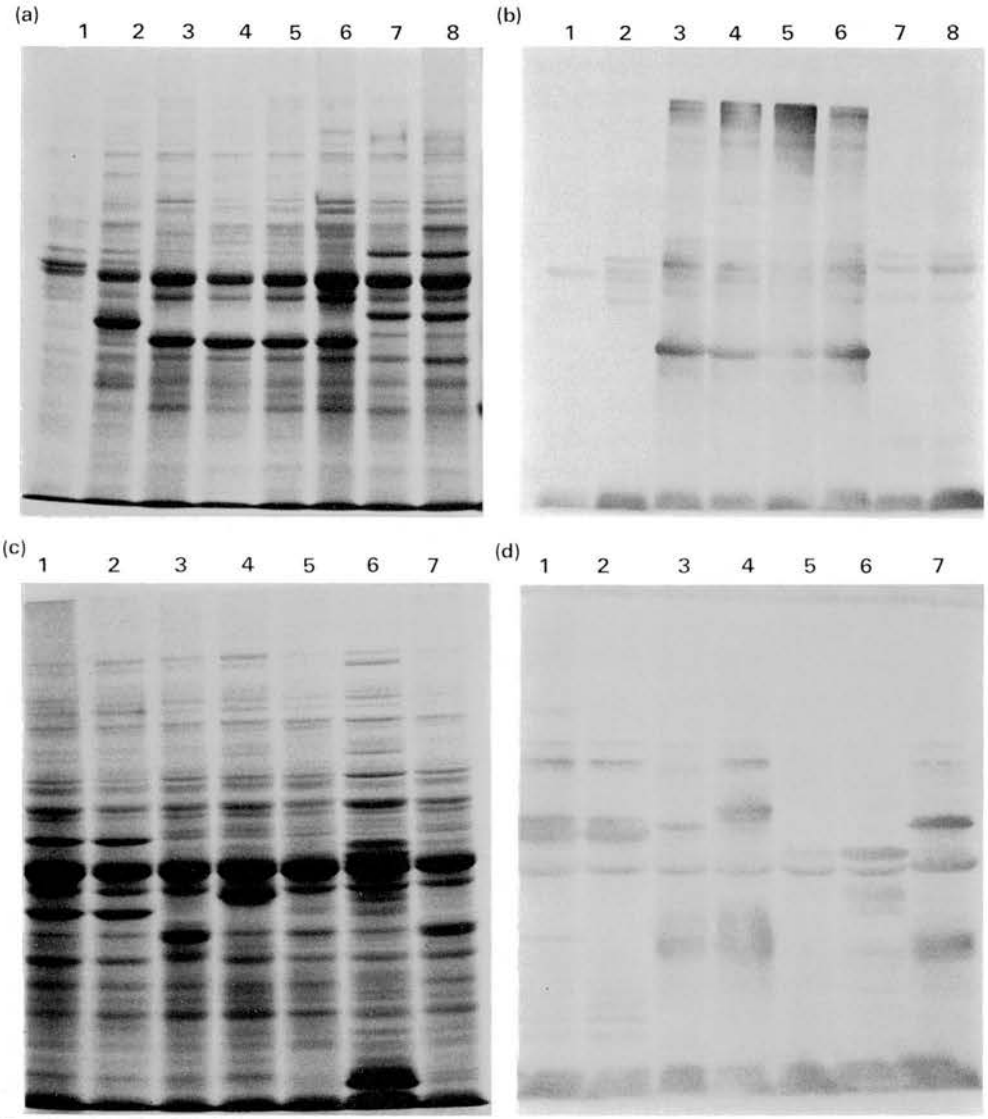

FIG. 2.—SDS-PAGE of EDTA extracts of 15 strains of *C. difficile* on 10% gels, and their corresponding electroblot transfers probed with NCTC11223 antiserum. Approximately 45 μg of protein in 50 μl of sample buffer was run on each track. (a) Coomassie Blue-stained SDS-polyacrylamide gel: track 1, group-3 pattern; track 2, group-5 pattern; tracks 3-6, group-1 patterns; tracks 7 and 8, group-2 patterns. (b) Electroblot transfer of fig. 2a. (c) Coomassie Blue-stained polyacrylamide gel: tracks 1 and 2, group-2 patterns; tracks 3-7, ungroupable patterns. (d) Electroblot transfers of fig. 2c.

patterns had been produced overall. One pattern was produced by 18 strains (group A). The rest of the strains (group B) gave a different pattern (table II). Examples of the two patterns are shown in fig. 1. Several of the precipitin lines were shared by both groups, but the group-B pattern had a characteristic, strongly staining, slow-moving line (no. 1). The precipitin lines are numbered as in our previous publications (Poxton and Byrne, 1981; Poxton, 1982). It should be noted, however, that in this study some extra, weakly staining lines have been seen for the first time. This is a consequence of using undialysed EDTA antigens. Extensive freezing and thawing also resulted in the destruction of these antigens. The best results were obtained when antigens were used immediately after preparation, without freezing and thawing.

In the Coomassie Blue-stained polyacrylamide gels, many common bands were present, with identical patterns in some tracks. Duplicate antigen preparations gave identical patterns. Groups of similar patterns could be assembled with some difficulty, but the electroblot transfers immediately revealed groups of similar patterns. Fig. 2 shows two sets of SDS-PAGE patterns and their corresponding electroblots. When analysed in detail, the groups obtained with both techniques were identical (table II). Although some of the patterns produced by other strains were similar to those in groups 1–5, none were identical (group A6, table II). Others were considerably different and unlike any other (group A−, table II).

Comparison of the groups identified by CIE with those based on SDS-PAGE or electroblot analysis shows that group B and group 1 are identical. The isolates belonging to this group were, with one exception, obtained from the orthopaedic ward at Huddinge Hospital during the time of the outbreak of colitis and diarrhoea. Isolates obtained from other sources in Sweden were different from the isolates in the B1 group (table II). These data justify the assumption that the B1 isolates belonged to the same strain of *C. difficile* which spread within this ward, colonising five patients and causing severe colitis in another four patients. However, isolates from two patients with diarrhoea in the ward during the 14-day period did not belong to the B1 group. Thus, more than one strain of *C. difficile* was present in the ward during the period. Isolates sampled after and before the outbreak in this ward were found to be different from the B1-group strain of *C. difficile*.

None of the stool samples obtained from staff members or from the environment in the orthopaedic ward yielded *C. difficile* or its toxin.

Discussion

Studies with a selective medium have shown that there is a very low carrier rate for *C. difficile* in a healthy adult population (Aronsson *et al.*, unpublished observations). It has been suggested that such carriage is transient (Larson *et al.*, 1978). However, the immediate environment of infected patients has been found to be contaminated (Mulligan *et al.*, 1980; Fekety *et al.*, 1981), and spread of the organism from the environment and from person to person has been suggested (Kim *et al.*, 1981; Kim, Dupont and Pickering, 1983). Isolation of *C. difficile* has also been reported from household pets (Borriello *et al.*, 1983), seals, donkeys, camels, sewage, mud and sand (Hafiz and Oakley, 1976) and recently from a Kodiak bear with pseudomembranous colitis (Orchard, Fekety and Smith, 1983). Thus, exogenous sources of *C. difficile* are being increasingly recognised.

Our investigation is the first time that strains of *C. difficile* isolated from an outbreak of *C. difficile* colitis have been fingerprinted immunochemically and shown to be identical, and also different from other isolates in a control group (Wüst *et al.*, 1982). With only one exception, the strains isolated from this 14-day outbreak were identical with each other and different from other strains, as determined by CIE or SDS-PAGE-electroblot methods. Strains isolated before and after the outbreak from the same ward were also different.

In our experience, strains from diverse sources can have similar CIE profiles and it is only when striking differences occur, as in this study, that this method proves useful for typing. SDS-PAGE can be used alone; the patterns produced, however, are complex and are difficult to analyse without the use of specialised equipment, especially if tracks from different gels have to be compared. Electroblot transfer immunoblotting has proved extremely satisfactory. The technique is simple to perform in apparatus that can be made easily in the laboratory. The simplicity of the patterns allows straightforward visual analysis, and we conclude that investigations into the epidemiology of *C. difficile* should be considerably aided by this technique.

Our data strongly suggest that the same strain of *C. difficile* spread within a hospital ward, infecting 38% of the patients and causing disease in susceptible patients. It is not likely that this finding represents an overgrowth of endogenous *C. difficile* in these patients, but we have not been able to determine the means of transmission in this outbreak.

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An Immunochemical Method for Fingerprinting *Clostridium difficile*

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The use of sodium dodecyl sulphate-polyacrylamide gel electrophoresis in association with electrophoretic transfer of proteins to nitrocellulose and subsequent probing with antisera appears useful as a method for fingerprinting *Clostridium difficile*. Thorough testing of the stability of the antigenic nature of isolates of the organism during subculture and antigen preparation has shown it to be remarkably stable both in vitro and in vivo. Minor differences in the method of antigen extraction do not markedly alter the immunoblot patterns produced. It has also been demonstrated that an individual may harbour more than one strain of the organism at any one time. Results show the possible usefulness of this technique in studying the epidemiology of diarrhoeal disease known to be associated with *C. difficile*. It is suggested that for any serious study several colonies should be subcultured from the primary isolation plate.

Key words: *Clostridium difficile* – *immunoblotting* – *fingerprinting*

Introduction

Clostridium difficile is known to be associated with nearly all cases of antibiotic-associated pseudomembranous colitis. The organism also appears to be involved to some extent in a broad spectrum of other bowel conditions such as antibiotic-associated diarrhoea, post-operative diarrhoea and chronic inflammatory bowel disease. It has also been isolated from cases of sporadic non-antibiotic-associated diarrhoea (British Medical Journal, 1981; Brettle et al., 1982). Isolation of *C. difficile* from healthy adults is uncommon although the organism can be cultured from a high percentage of normal infants (George et al., 1977; Stark et al., 1982). The epidemiology of *C. difficile*-associated disease is poorly understood at present (Mulligan, 1984). There have been several reports indicating possible cross-infection between patients from whom the organism has subsequently been isolated. However lack of a reliable typing technique has hindered more detailed studies of these outbreaks. Sell et al. (1983) suggested that the use of bacteriophage could prove useful in typing of *C. difficile* while Tabaqchali et al. (1984) have used radiolabelling of growing cells as a possible means of studying the epidemiology of the organism. Since then Delmee

et al. (1985) have shown that strains of the organism can be serogrouped into 6 different groups using a variety of antisera. Recently, in this laboratory the use of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in association with electrophoretic transfer of proteins to nitrocellulose and subsequent probing with antisera has provided a useful method for fingerprinting the organism (Poxton et al., 1984). Further studies have subsequently been performed to test the reproducibility of results obtained with this technique. This paper provides a review of the experimental work carried out and offers guidelines for the successful application of the technique in the clinical situation.

Materials and Methods

Bacterial isolates

Isolation and identification of *C. difficile* was done as previously described (Brettle et al., 1982). Four strains of the organism, MPRL 558, 559, 589 and 720, which had been isolated from patients attending the Renal Unit in the Royal Infirmary of Edinburgh (RIE) during 1983 were used in this study. They were picked from the primary isolation plates as single colonies, cultured overnight in cooked meat broth (CMB) and lyophilised. MPRL 558 came from a severely ill patient; MPRL 559 from a patient with no diarrhoea while MPRL 589 and MPRL 720 came from 2 different patients with moderate diarrhoea.

Standard technique for culture and isolation of surface protein antigens

Lyophilised organisms were cultured in CMB for 17 h and 0.1 ml of this was inoculated into 100 ml proteose peptone yeast extract broth (PPY) supplemented with 0.04% (w/v) sodium carbonate and 0.075% (w/v) cysteine hydrochloride as described by Poxton et al. (1984). After 17 h incubation anaerobically at 37°C the cells were harvested ($20{,}000 \times g$ for 5 min at 4°C) and the washed pellet was resuspended in phosphate-buffered saline, pH 7.4, containing 10 mM EDTA and incubated at 45°C for 30 min. The supernatant, containing the antigens, was collected after 2 cycles of centrifugation at $10{,}000 \times g$ for 2.5 min and used undialysed as antigen. The protein content of the preparation was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Enrichment broth and selective agar

C. difficile was isolated from mouse faecal specimens using cycloserine-cefoxitin egg yolk agar (CCFA) (George et al., 1979). Enrichment was achieved using CCF broth which was CCFA without the egg yolk and agar.

SDS-PAGE

The buffer system of Laemmli (1970) with 10% slab gels as described by Poxton and Brown (1979) was used. EDTA extracts containing 25 μg of protein in 50 μl of sample buffer were run on gels in duplicate. One set of the separated proteins was stained with Coomassie blue and the other was used for transfer to nitrocellulose membrane.

Electroblot transfer

The method of Towbin et al. (1979) as described by Poxton et al. (1984) was followed. This involved transfer of the SDS-PAGE-separated EDTA extracts to nitrocellulose membrane in a Tris, glycine, methanol buffer pH 8.3 at 12 V and 40 mA for 18 h. After this the membrane was washed and probed with a rabbit antiserum raised against UV-killed cells of *C. difficile* NCTC 11223. The method of raising the antiserum was as described by Poxton and Byrne (1981).

Results

Work carried out with the standard technique for fingerprinting of *C. difficile* isolates which was described earlier (Poxton et al., 1984) has raised questions as to the reproducibility of the immunoblot patterns produced from one study to the next. Minor variations occurred between blotting patterns produced from different clinical isolates. It was important to find out whether these differences were due to real variations between strains or whether they arose simply because of differences in growth conditions of particular strains or variation in antigen extraction. These studies were therefore done to investigate the antigenic stability of isolates, both during culture of the organism and subsequent antigen extraction.

Effect of variation in phase of growth at harvesting

In the standard technique the organisms were cultured for 16–17 h before harvesting and antigen extraction. In order to find if culturing of the isolates for differing periods would produce differences in the resulting immunoblot patterns obtained, the following experiment was done. A 5% inoculum of MPRL 558 put into PPY medium was incubated anaerobically at 37°C and 100 ml amounts were harvested after 2, 4 and 8 h. Similarly a 1% inoculum was harvested after 16, 18, 20, 24 and 40 h. All other conditions remained the same throughout. It can be seen from the resulting immunoblot patterns produced (Fig. 1a) that there was a great degree of similarity in the banding patterns. There were minor variations, especially in the higher molecular weight polypeptides, but these may in part be due to slight variations in the amount of protein actually loaded onto the gel as seen in the corresponding Coomassie blue stain (Fig. 1b).

Variations in antigen extraction

Another likely source of variation between isolates could be differences in the actual method of antigen preparation. To investigate this 3 of the renal isolates were grown up in PPY for 17 h, harvested, washed and resuspended in EDTA buffer as described previously. The EDTA suspensions were then divided into 4 equal aliquots and each was subjected to one of the following treatments, (a) 'normal' antigen extraction at 45°C for 30 min, (b) extraction at 50°C for 30 min, (c) extraction at 45°C for 2.5 h, (d) freeze-thawing (− 20°C to 37°C) 3 times over a period of 3 h and then 45°C for 30 min. These 4 EDTA suspensions were then centrifuged as described in Materials and Methods.

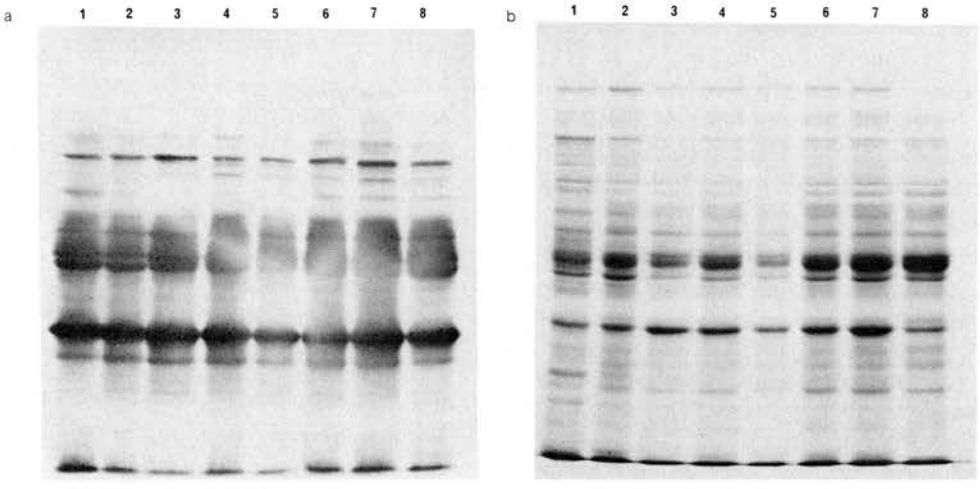

Fig. 1. (a) Immunoblot patterns produced by EDTA extracts of *C. difficile* MPRL 558 obtained 2–40 h after inoculation of PPY medium. Tracks 1–8 contained extracts obtained after 2, 4, 8, 16, 18, 20, 24 and 40 h respectively. Extracts were probed using antiserum raised to NCTC 11223. (b) Corresponding SDS-PAGE profile stained with Coomassie blue.

When these various extracts were run on gels it was found that MPRL 558 and 559 produced very similar banding patterns by Coomassie blue staining but there were consistent differences between the isolates in the patterns produced on immunoblotting (Fig. 2a and 2b). There was a much stronger reaction involving the high molecular weight proteins in MPRL 559 than occurred with MPRL 558 and this remained consistent despite the differing treatments of the extracts during extraction. However, MPRL 589 behaved differently. It was found that the extracts which had been heated for 2.5 h produced a blot pattern which was different from the others in the mid-region of the track (Fig. 2c). Why this occurred is not clear. With all 3 isolates the EDTA/freeze-thawed extracts had more protein bands apparent on the Coomassie stained gel. However, the immunoblot patterns obtained did not show up these additional bands indicating that they were probably intracellular proteins with which the antisera raised against whole UV-killed bacteria would not react.

Variations on subculture of the organisms

Having found that no major variations in immunoblot patterns could be accounted for by differences in culture or antigen preparation, further work was done to investigate the stability of an individual isolate both in vitro and in vivo. MPRL 558 was used for both these areas of investigation. A CMB culture of the isolate was passaged weekly into fresh pre-steamed CMB over the course of 7 weeks. An EDTA extract of each of these broths was prepared in the standard way after each had been incubated anaerobically at 37°C for 24 h. When these extracts were analysed by SDS-PAGE and immunoblotting the patterns produced were identical.

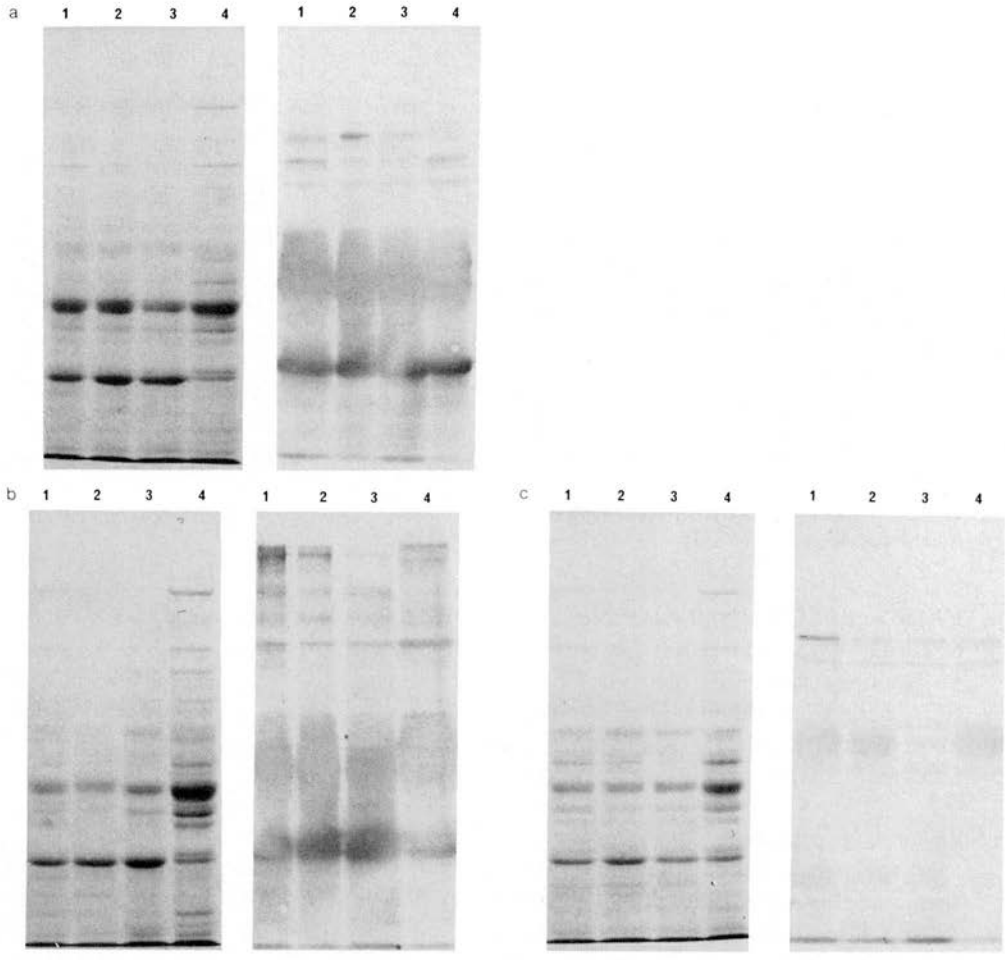

Fig. 2. SDS-PAGE and immunoblot patterns produced by (a) MPRL 558, (b) MPRL 559 and (c) MPRL 589 after various treatments during EDTA extraction. Track 1: extraction at 45°C for 30 min, track 2: 50°C for 2.5 h, track 3: 45°C for 2.5 h, track 4: freeze-thawed extract.

To study antigenic variation of the organism in vivo a spore suspension of MPRL 558 (100 μl saline inoculated with 10 *C. difficile* colonies from a 72 h blood agar plate) was given orally to a female BALB/c mouse which was known to be *C. difficile*-negative. Faecal samples were collected weekly from the animal over a period of 7 weeks. Each sample was inoculated into enrichment broth and the organisms were recovered by plating of these broths onto CCFA. The isolates were stored as CMB cultures and an EDTA extract of each isolate was prepared by the standard method. Again it was found that there were only minor variations between each of the isolates.

Another study was done with 3 strains where one colony from a CCFA plate was streaked onto blood agar. Eight colonies were subsequently picked from this into

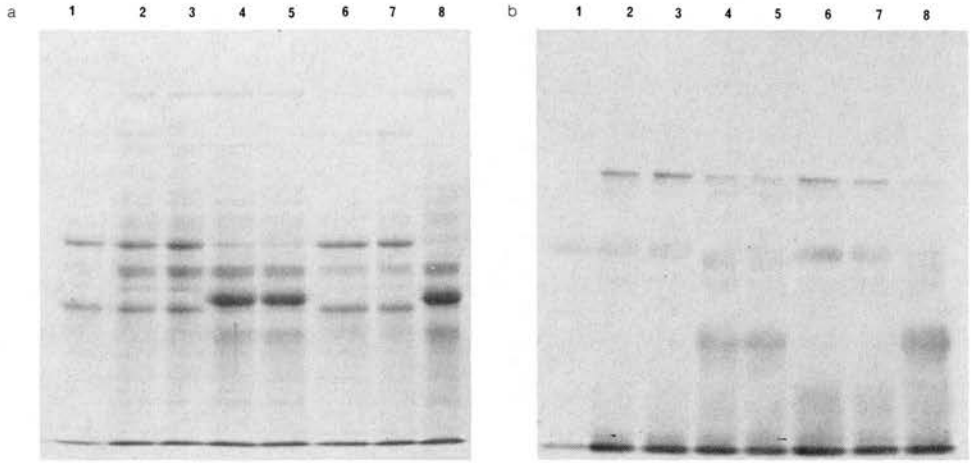

Fig. 3. Analysis of EDTA extracts prepared from 8 different colonies picked from a CCFA primary isolation plate. (a) SDS-PAGE, (b) immunoblot.

CMB and EDTA extracts prepared of each colony. When these extracts were analysed it was found that the patterns produced were identical.

The only other possible source of variation during this work was in the composition of the PPY medium used to culture the organisms. However, these studies were done over a period of 4 months during which several different batches of media were used and no variation in antigenic patterns was observed.

Different strains within one patient

It was now apparent that any one strain of *C. difficile* would remain antigenically the same throughout any study. Further work was consequently done to see whether an individual patient could harbour more than one strain of the organism at one time. The original faecal sample from which MPRL 720 had been isolated was spread onto CCFA. After incubation 8 different colonies were subcultured from this plate. EDTA extracts were prepared from all of these isolates which were subsequently analysed. It was found that from this primary isolation plate 2 different banding patterns were produced by the isolates (Fig. 3). The pattern produced in tracks 1, 2, 3, 6 and 7 was as expected for MPRL 720 (as indicated by other studies); the pattern in the other tracks corresponding to that of another isolate cultured from this patient 6 months previously. When this experiment was repeated with another 2 faecal samples one of these was also found to contain 2 different strains of *C. difficile*.

Discussion

The immunochemical fingerprinting of *C. difficile* strains described here has been used in this laboratory over the past 2 years to study various outbreaks of diarrhoea

with which the organism was found to be associated. The first investigation involved studying 28 strains of *C. difficile* sent from an outbreak in Sweden (Poxton et al., 1984). With this technique it was possible to show the existence of a common strain within one area of a hospital. The results of this study showed that this technique could prove useful in tracing the epidemiology of *C. difficile*. Since then a further study has been done involving renal patients attending the RIE who had differing degrees of diarrhoea. In this particular group of 18 patients 13 different strains of the organism appeared to be associated with their symptoms over a 9-month period (Cumming et al., unpublished results). It was found however that in one or two instances it was difficult to be absolutely certain whether or not 2 strains were the same or different. For this reason it was felt necessary to investigate the stability of the antigens that were being isolated and studied with this technique.

As we know from our previous studies, the Coomassie blue and immunoblot patterns vary considerably from strain to strain although one or two major bands and several minor bands are common to all strains. Some of the major bands in Coomassie blue gels are antigenic while others are not, and this is also true for the minor bands. In this study the variation in antigenicity of the bands can be seen by comparing the patterns produced in Fig. 1 and 3. In Fig. 1 most of the major Coomassie blue bands are also stained strongly in the immunoblot, while in Fig. 3 only one of the major Coomassie blue bands in tracks 1, 2, 3, 6 and 7 is strongly immunogenic and most of the staining of the immunoblot is due to minor bands.

It is apparent from the studies done here that the antigenic nature of any particular isolate of *C. difficile* will remain constant when analysed by this immunoblotting technique. The only major difference observed in an isolate was with MPRL 589 when it was left to incubate for an extended period of time during EDTA extraction of the antigens. It has been noticed that variations in the intensity of banding seen on the Coomassie stain can lead to difficulty in interpreting the immunoblotting patterns. Protein bands that are faint often do not show up on the immunoblot. However, these minor problems do not detract from the usefulness of this technique in the epidemiology of any outbreaks of diarrhoeal disease known to be associated with *C. difficile*.

Recommendations for successful use of immunoblotting

(1) Pick several colonies (at least 10) from the primary isolation plate in case there is more than one strain of *C. difficile* present in the patient.

(2) A standardised technique should be used for the culture and preparation of the EDTA antigen extracts. Cells should be incubated for 30 min and at a temperature of 45°C although minor variations in this do not appear to matter. Since different strains may vary in their sensitivity to changes during antigen extraction it is best to keep to a well-defined regimen. Freezing and thawing during the EDTA extraction procedure releases more protein: this complicates the Coomassie blue pattern, adds nothing to the immunoblot and should be avoided.

(3) Only compare EDTA antigens run on the same gel. It is difficult to compare one gel with another as it is impossible to reproduce running conditions accurately.

(4) When analysing the SDS-PAGE and immunoblot patterns produced by this

technique minor variations should be overlooked if the general picture arising is of similarity between the strains.

(5) Conclusions should be drawn using the information provided by both the gel and the immunoblot since some patterns that appear similar on the blot can appear quite different on the gel and vice versa. The advantage of doing an immunoblot as well as a Coomassie blue-stained gel is that the blot is simpler and easier to read.

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Diarrhoea due to Clostridium difficile associated with antibiotic treatment in patients receiving dialysis: the role of cross infection

Diarrhoea due to *Clostridium difficile* associated with treatment with antibiotics has been described among patients receiving peritoneal dialysis,[1] and cross infection is thought to be important.[2] We describe an outbreak of diarrhoea associated with *C difficile* in patients undergoing haemodialysis and continuous ambulatory peritoneal dialysis in which a "fingerprinting" technique of typing strains was used to investigate the possibility of person to person spread.

Patients, methods, and results

The table gives details of 18 patients from whom *C difficile* was isolated on stool culture. All developed diarrhoea while inpatients in the medical renal unit, Royal Infirmary, Edinburgh, between July 1983 and April 1984. *C difficile* had been isolated from only one patient with renal disease in the previous six months.

C difficile was cultured and identified as previously described[3]; strains were identified by the fingerprinting method of Poxton *et al*, using SDS-polyacrylamide gel electrophoresis of surface proteins extracted with edetic acid followed by Coomassie blue staining and an immunoblot probe using rabbit antiserum to cells of *C difficile* NCTC 11223 killed with ultraviolet light.[4] When *C difficile* was isolated patients were given oral vancomycin (500 mg every six hours) and other antibiotics were withdrawn if possible. Diarrhoea resolved in 12 patients. Four patients died during or shortly after treatment; all were severely debilitated by pre-existing medical conditions. The fingerprinting technique identified 13 different strains of *C difficile*. One strain occurred in five subjects (cases 12, 13, 14, 15, and 18) and one strain in two (cases 7 and 11); the 11 other strains occurred in only one patient each.

Comment

Cross infection with *C difficile* in hospitals has been clearly shown previously,[4] and seemed likely in this series of cases among our patients receiving dialysis; all had been inpatients in the medical renal unit, with considerable overlap in their periods of stay in hospital, and the rate of isolation of *C difficile* increased abruptly over 10 months. Standard measures to prevent spread of the organism were taken—namely, isolation when feasible, use of gown and gloves when working with patients, and careful attention to personal hygiene.

Isolation of patients was limited by lack of space and the specialised nursing that dialysis requires. The five patients from whom the same strain was isolated were probably cross infected; all were nursed in one of two adjacent cubicles, the first four within one month. The isolation of 13 different strains of *C difficile* appears, however, to exclude cross infection as the major mechanisms by which organisms were acquired during this outbreak. Among patients undergoing dialysis who have uraemia the frequent use of broad spectrum antibiotics, defective immunity, abnormal nutrition, and perhaps other changes in gut flora or mucosal defence mechanisms might combine to permit acquisition of *C difficile* or to promote its selective growth.[5] After this outbreak we tried to give as narrow a range of antibiotic treatment as possible and avoided oral antibiotics, particularly oral cephalosporins; the incidence of isolation of *C difficile* and related clinical disease returned to a low level.

We recommend early selective faecal culture for *C difficile* in any patients undergoing dialysis who have diarrhoea. Our findings suggest that cross infection with *C difficile* may occur in patients receiving dialysis, although it is not always the major mechanism of acquisition of this organism. It would be unwise to abandon standard measures against cross contamination, and it should be appreciated that patients undergoing dialysis may be particularly prone to infection with *C difficile*.

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Details of patients from whom C difficile was isolated

Case No	Age (years)	Sex	Type of dialysis	Type of infection	Antimicrobials given	Month when strain isolated	Outcome
1	61	F	CAPD	Peritonitis	None	July 1983	Resolved
2	60	F	CAPD	Peritonitis	Cephradine, flucloxacillin, tobramycin	July 1983	Died
3	48	F	Haemodialysis	None	None	July 1983	Remained well
4	15	F	CAPD	Peritonitis	Cephradine, tobramycin	August 1983	Died
5	56	F	Haemodialysis	Wound	Cefuroxime, metronidazole	August 1983	Diarrhoea continued
6	59	F	Haemodialysis	Arteriovenous fistula	Flucloxacillin, benzylpenicillin	August 1983	Resolved
7	61	M	CAPD	Peritonitis	Flucloxacillin, metronidazole, ticarcillin	August 1983	Resolved
8	69	F	CAPD	Peritonitis	Cephradine	September 1983	Resolved
9	59	M	Haemodialysis	Mastoid	Flucloxacillin, benzylpenicillin	October 1983	Resolved
10	50	M	Haemodialysis	Pericolic abscess	Cephradine, cefuroxime, metronidazole	October 1983	Resolved
11	68	F	CAPD	Peritonitis	Tobramycin	November 1983	Resolved
12	71	F	CRF	None	None	January 1984	Resolved
13	73	M	Haemodialysis (acute)	Pneumonia	Ampicillin, cefuroxime, erythromycin, metronidazole, gentamicin, benzylpenicillin	January 1984	Resolved
14	33	F	Haemodialysis	Urinary tract	Co-trimoxazole	February 1984	Resolved
15	63	F	Haemodialysis (acute)	Ischaemic bowel	Cefuroxime, metronidazole, tobramycin	February 1984	Died
16	64	F	CAPD	Peritonitis	Flucloxacillin	February 1984	Resolved
17	60	M	CAPD	Peritonitis	Flucloxacillin	March 1984	Resolved
18	66	F	Haemodialysis	Arteriovenous fistula	Cephradine, cefuroxime, tobramycin	March 1984	Died

CAPD=Continuous ambulatory peritoneal dialysis. CRF=End stage chronic renal failure.

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Typing of *Clostridium difficile* causing diarrhoea in an orthopaedic ward

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SUMMARY In an outbreak of diarrhoeal disease in an orthopaedic ward *Clostridium difficile* was isolated from all six patients with diarrhoea. Attempts were made to type these isolates by means of antibiogram, detection of pre-formed enzymes, analysis of surface proteins by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, and plasmid profile analysis. This showed that a single strain (type E) indistinguishable by the four distinct methods of typing, was isolated from all six patients at some time during their episodes of diarrhoea. Relapse was caused by the acquisition of a new strain in two patients, and by re-emergence or reacquisition of the original strain in two patients. The immunochemical method was the most sensitive and discriminatory of the typing strategies adopted.

Clostridium difficile was first implicated as the cause of pseudomembranous colitis in 1978.[1–3] Since then, numerous reports have documented that the organism can cause a broad spectrum of bowel disorders which include antibiotic-associated diarrhoea, chronic inflammatory bowel disease, and non-antibiotic associated diarrhoea.[4–8]

The role of *C difficile* in the pathogenesis of these conditions is still the subject of controversy. It has also been difficult to define the epidemiology because of the lack of reliable and readily available typing methods. Several methods have been described. These include antibiograms, analysis of surface proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, plasmid profiles, bacteriocin and bacteriophage typing, autoradiograms of ^{35}S methionine labelled proteins extracted from growing cells, and serogrouping by agglutination.[4,8–11] In this study we used a combination of antibiograms, analysis of pre-formed enzymes,[12] SDS-PAGE/immunoblotting and plasmid profiles to investigate an outbreak of diarrhoeal disease due to *C difficile* in an orthopaedic ward in Glasgow Royal Infirmary.

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Patients and methods

The six patients (mean age 79 years; range 72–88) were elderly women who had been admitted to an orthopaedic ward of Glasgow Royal Infirmary. Due to their underlying conditions the patients were all bedbound. Individual details are given in table 1.

Faecal samples were taken from those patients who developed diarrhoea. When the possibility of an outbreak was suspected faecal samples from all the patients in the ward, together with environmental samples from the ward floor, bedpan wagon, "clinimatic", toilets and from ward furniture, were cultured. In addition to the isolates from the "outbreak", five other strains of *C difficile* from sporadic cases of

Table 1 *Summary of clinical details*

Case No	*Sex*	*Age*	*Reason for admission*	*Previous use of antibiotics*	*Outcome*
1	F	88	Fractured neck of femur	Yes	Infectious diseases unit
2	F	79	Dislocated Thomson's prosthesis	Yes	Died
3	F	79	Dislocated Thomson's prosthesis	Yes	Infectious diseases unit
4	F	82	Bone graft, right femur	Yes	Infectious diseases unit
5	F	75	Fractured neck of femur	No	Convalesced
6	F	72	Fractured neck of femur	Yes	Infectious diseases unit

Convalesced—patient transferred to another orthopaedic ward.

diarrhoea in the hospital isolated during the previous six months were kept to be used as controls for surface protein analysis.

All samples were inoculated on to cycloserine cefoxitin agar (CCFA)[13] and incubated anaerobically at 37°C for 48 hours. Isolates were identified as *C difficile* by colonial morphology, characteristic smell, Gram stain, toxin production, biochemical reactions and by gas-liquid chromatography.[14] Faecal samples were also cultured on MacConkey agar, deoxycholate citrate agar (DCA), campylobacter agar and in selenite F broth for the presence of other enteric pathogens.

TEST FOR TOXIN PRODUCTION

Faecal samples were centrifuged directly at 2000 × *g* for 20 minutes or, if solid, first suspended in an equal volume of saline before centrifugation. The supernatant was filtered through a 0·45 μm membrane filter and then diluted 1 in 50 and 1 in 500 in tissue culture growth medium before inoculation on to Vero cell monolayers: the cells were then incubated for 18 hours at 37°C. The presence of toxin was indicated by the characteristic cytopathic effect. If a sample was found to be positive at the screening dilutions, the concentration of toxin present was established by testing serial doubling dilutions from 1 in 50 to 1 in 6400. The specificity of the toxin was confirmed by neutralisation with *Clostridium sordellii* antitoxin.[14]

ISOLATION OF SURFACE PROTEIN ANTIGENS

Organisms were cultured in cooked meat broth (CMB) overnight and 0·1 ml inoculated into 100 ml of protease peptone yeast extract broth (PPY), supplemented with 0·04% (w/v) sodium carbonate and 0·075% (w/v) cysteine hydrochloride as described by Poxton *et al.*[4] After overnight incubation anaerobically at 37°C the cells were harvested by centrifugation at 20 000 × *g* for five minutes at 4°C and the pellet resuspended in phosphate buffered saline, pH 7·4, with 10 mM ethylene diamine tetra-acetic acid (EDTA): the suspension was then incubated at 45°C for 30 minutes. The supernatant, which contained the concentrated antigens, was collected after two cycles of centrifugation at 10 000 × *g* for 2·5 minutes and used without dialysis as the antigen preparation. The protein content was measured by the method of Lowry *et al.*[15] using bovine serum albumin as standard.

ANALYSIS OF PROTEINS IN SDS-PAGE

The buffer system of Laemmli[16] with 10% slab gels was used as previously described by Poxton and Brown[17]. Extracts in EDTA which contained 25 μg of protein in 50 μl of sample buffer were run in duplicate on gels. One set of the separated proteins was stained with Coomassie blue and the other was used for transfer to nitrocellulose membrane.

IMMUNOBLOT TRANSFER

The method of Towbin *et al*[18] was used. SDS-PAGE separated EDTA extracts were transferred to nitrocellulose membrane in a Tris, glycine, methanol buffer, pH 8·3, at 12 V and 40 mA for 18 hours. The membrane was then washed and probed with a rabbit antiserum raised against cells of *C difficile* (NCTC 11223) killed by ultraviolet light followed by application of an antirabbit IgG-horseradish peroxidase (HRP) conjugate and appropriate colour reagent (BioRad).[19]

DETERMINATION OF PLASMID PROFILES

Overnight anaerobic cultures (18 hours) in pre-reduced brain heart infusion broth at 37°C were prepared. The cells were harvested by centrifugation at 2000 × *g* for five minutes, and the pellets resuspended in 200 μl of Tris, EDTA, sodium chloride (TES) dissolved in 50 mM sucrose to which 50 μl of lysostaphin (1 mg/ml) and 50 μl of lysozyme (40 mg/ml) were added. After incubation for 45 minutes at 37°C 80 μl of 0·25 M EDTA was added before incubation at 56°C for 30 minutes, after which 400 μl of 10% w/v sodium dodecyl sulphate was added to complete lysis. Crude lysates were centrifuged for 15 minutes at 15 000 × *g*, and the supernatants run on 0·7% agarose gels, as described previously.[20,21] Plasmids of known size were used as molecular weight standards. A strain of *C difficile* known to harbour an 80 megadalton plasmid was processed in parallel as a positive control.

ANTIBIOGRAM TYPING

Antibiogram patterns were determined by estimation of minimum inhibitory concentrations (MICs) of erythromycin, tetracycline, chloramphenicol, rifampicin and clindamycin. This was done by an agar dilution method with two-fold dilutions of antibiotic incorporated in Wilkins-Chalgren agar.[9,22] The antibiotic concentrations tested ranged from: erythromycin 1 to 64 mg/l; tetracycline 0·25 to 32 mg/l; chloramphenicol 8 to 128 mg/l; rifampicin 0·25 to 256 mg/l and clindamycin 8 to 256 mg/l. Plates were inoculated with about 100 000 organisms/ml and incubated anaerobically overnight at 37°C. Sensitive strains were defined as those that did not grow at the lowest antibiotic concentration, and resistant strains as those which were not inhibited by the highest concentration tested.

ENZYME PROFILES

Pre-formed enzymes were detected by the API ZYM system. Overnight broth cultures of each isolate were centrifuged and the pellet suspended in 2 ml sterile

Table 2 *Comparison of antibiogram pattern and immunochemical type*

Case No	Date	Strain	Antibiogram					Anti-biotype	Immuno-chemical type
			Erythromycin	*Chloramphenicol*	*Clindamycin*	*Rifampicin*	*Tetracycline*		
1	28/4	Primary	R	S	S	S	S	2	D
	20/5	Relapse	R	S	R	S	S	1	E
2	22/5	Primary	R	S	R	S	S	1	E
	26/5		R	S	R	S	S	1	E
3	26/5	Primary	R	S	R	S	S	1	H
	30/5		R	S	R	S	S	1	E
	7/6	Relapse	R	S	R	S	S	1	E
	9/6		R	S	R	S	S	1	E
4	26/5	Primary	R	S	R	S	S	1	E
	19/6	Relapse	R	S	S	S	S	2	A
5	4/6	Primary	R	S	R	S	S	1	E
	7/6		R	S	S	S	S	2	D
6	6/6	Primary	R	S	R	S	S	1	E
	10/6		R	S	R	S	S	1	E
	25/6	Relapse	R	S	R	S	S	1	E

R = resistant; S = sensitive.

water to obtain a suspension with a turbidity which measured between McFarland values No 5 and No 6. Volumes (65 μl) of this suspension were then inoculated into the 20 cupules of the test strip (one control + 19 tests) and incubated in darkness at 37°C for four hours.

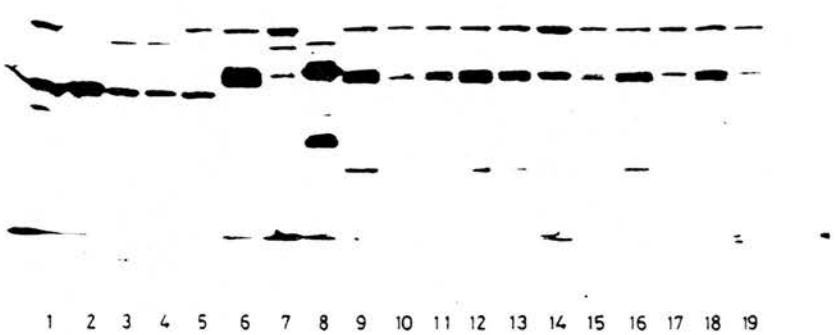
The test strips were processed, and the results interpreted in accordance with the manufacturer's instructions. Each isolate was tested on two separate occasions.

Results

In late May and early June 1987 six patients in the orthopaedic ward of the Glasgow Royal Infirmary were involved in an outbreak of diarrhoea (table 2). Three weeks earlier, one patient (case 1) had required treatment for an isolated episode of diarrhoea associated with *C difficile*. This patient was treated with vancomycin for 10 days with good clinical response but five days later she again developed diarrhoea. Over the next 17 days five other patients had episodes of diarrhoea due to *C difficile*.

Five of the six patients in the outbreak had received antibiotics within the previous four weeks. These included flucloxacillin (four patients), ampicillin and amoxycillin (two patients), fusidic acid (one patient), erythromycin (one patient) and cephalexin (one patient).

All patients were treated with oral vancomycin. One



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Fig 1 *Initial 19 strains typed by immunochemical method (14 from outbreak: 10 patients, four environmental, five from elsewhere in hospital).*

Lanes: 1 A; 2 B; 3, 4 C; 5 G; 6 H; 7 D; 8 F; 9–19 E.

Fig 2 *Eight different stains A–H. Strains A, D, H, E isolated from patients in "outbreak". Strains B, C, G, F from elsewhere in hospital.*

Lanes: 1 A; 2 B; 3 C; 4 G; 5 H; 6 D; 7 F; 8 E.

patient (case 2) died. The cause of death was not directly related to *C difficile* diarrhoea, although this may have been a contributory factor. Of the other five patients, four had further episodes of diarrhoea from which *C difficile* was isolated. The outbreak was eventually controlled by closure of the ward to further admissions and transfer of those patients with relapses to the local infectious diseases unit.

C difficile was isolated from the six patients on the ward (in some cases on more than one occasion) and from the bedside locker, bedpan holder, and surrounding floor of one of the affected patients and from the bedside locker of an asymptomatic patient in the ward. *C difficile* was not isolated from any of the patients without diarrhoea. No other enteric pathogens were isolated from any patient.

The 15 isolates of *C difficile* from the six patients and the four environmental isolates produced toxin. All faecal samples from the six patients were also found to contain high titres (> 1 in 6400) of *C difficile* toxin.

Antibiotic sensitivity tests separated the isolates into two "antibiotypes" on the basis of clindamycin sensitivity. Type 1 was resistant to clindamycin and type 2 was sensitive. All the environmental samples were "antibiotype" 1. A comparison between antibiogram patterns and immunochemical types in the patient samples is shown in table 2.

None of the *C difficile* isolates studied harboured plasmids.

The following enzymes were present in all isolates from patients and the environment: alkaline phosphatase, esterase (C4), esterase lipase (C80), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase.

Nineteen strains of *C difficile* from the outbreak and five sporadic isolates from elsewhere in the hospital were submitted with no indication of source for SDS-PAGE/immunoblot fingerprint analysis. Eight immunochemical types, arbitrarily designated A–H, were found (figs 1 and 2). Four of these, A, D, E, and H, were found only in samples from the "outbreak". The other four patterns, B, C, F, and G were produced from the five strains isolated from elsewhere in the hospital. Strains with pattern E were isolated at least once from all the affected patients and from the surrounding environment at some time during their episodes of infection. Types A, D, and H were only found sporadically and were present only in single samples from four different patients (table 2).

Discussion

Results of this study suggest that a single common strain (type E) of *C difficile*, isolated from all six patients and from the environment, was almost certainly responsible for the outbreak of diarrhoea.

These results support the observations of other workers[8 9 11 23] that *C difficile* can be responsible for nosocomial infection in the hospital environment, especially in elderly debilitated patients. It is clear from this study that diarrhoea can relapse after treatment with an appropriate agent (vancomycin) and that the same strain may again be implicated. The reasons for such relapses are not clear but for the first time we have shown that relapse can be caused by acquisition of a new strain (cases 1 and 4) or by re-emergence, or reacquisition of the original strain (cases 3 and 6).

The immunochemical method was shown to be the most discriminatory of the typing strategies evaluated. Antibiograms showed the presence of two types, distinguishable only by their clindamycin sensitivity. Type 1 (clindamycin resistant) was further divided into the "outbreak" type E and another strain, type H, by immunochemical methods. Type 2 (clindamycin sensitive) was further divided into types A and D by immunochemical methods.

The presence of four strains in the outbreak complicated the epidemiological investigation. Although not as complex as a recent outbreak in a renal unit,[24] this outbreak shows that multiple strains of *C difficile* may be involved in a susceptible patient population.

Ideally, 10 colonies should be picked from primary isolation plates for immunochemical analysis[25]

because more than one strain may be present in primary cultures from individual patients. Unfortunately, this was not done in this study, because it was not at first clear that we were dealing with an outbreak. A similar caveat, however, could equally be applied to many other epidemiological studies of outbreaks which have used this and other typing strategies. Although types A, D, and H were isolated from four of the patients at some time during their diarrhoeal episodes, type E might well have been present on the primary isolation plate.

Levett[12] used the API ZYM system to identify *C difficile* from other clostridial species. He noticed that a few strains had other enzymes present in addition to the common enzymes found in all the *C difficile* strains tested. We found that all our isolates produced the same enzymes and that this result was reproducible. Consequently this method is unlikely to be useful as a typing method for *C difficile*, unless an unusual enzyme pattern is found.

Plasmid profile data have been used to provide useful epidemiological information in outbreaks of infection caused by a variety of different bacterial genera.[26] Most strains of *C difficile*, however, do not harbour plasmids and it is well recognised that absence of plasmids or very limited plasmid diversity limits the usefulness of this technique as an epidemiological tool.

Peerbooms *et al* have described the use of restriction endonuclease digest patterns of *C difficile* chromosomal DNA as a means of typing,[27] and this method may find wider application in the future.

Immunochemical and plasmid profile analysis can be undertaken in a labortory with standard electrophoresis equipment, but technical expertise is required to operate these systems successfully, so that these techniques are likely to be restricted to specialist centres. "Antibiotyping" is much less technically demanding and is probably a useful initial step in the investigation of an outbreak but a more discriminating technique should also be used.

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Current Approaches to the Classification, Characterisation and Typing of Pathogenic Anaerobic Bacteria

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ABSTRACT. Characterisation systems based on the demonstration of soluble antigens and toxins are of much practical value but have clear taxonomic limitations. This is illustrated by reference to our work with some of the pathogenic clostridia. The definition and classification of the anaerobic cocci is a difficult and continuing challenge. Systems for the classification and characterisation of the gram-negative non-sporing anaerobic bacilli are evolving well, though there are still some very difficult areas. A selection of the most helpful tests is indicated. Biotyping systems are compared with serotyping systems; the results of serological approaches to characterisation are encouraging. The further exploration of bacteriocin typing seems to be merited. The challenge of characterisation and classification of anaerobic spirochaetes commonly involved in human infections must now be taken up.

INTRODUCTION

So much work is being done on the anaerobic bacteria that it is helpful to consider practical aspects of classification and characterisation in 1981, to update our views expressed in 1979 (1). In this paper, we review significant trends and we consider results of our recent studies of various pathogenic anaerobes, with special reference to practical approaches to their prompt characterisation and effective classification in relation to medical and dental bacteriology.

MATERIALS AND METHODS

Organisms: the strains studied are detailed in recent papers published by past and present members of our research team; the relevant references are given in the text. Biochemical tests were as described previously (5, 6). Gas liquid chromatography: The procedures used were described by Deacon and colleagues (4). Neuraminidase assays were performed as described by Fraser and Brown (8, 9). Bacteriocin studies were as outlined by Watt and Collee (22).

RESULTS AND DISCUSSION

The clostridia. Two organisms provide interesting examples of challenges to our existing systems of classification and characterisation. These are *Clostridium perfringens* and *C. difficile*.

C. perfringens. Although strains of types A1 and A2 replicate very rapidly at 44–45°C with mean generation times of less than 10 min (2, 3) we found that production of lecithinase (phospholipase-C) is suppressed at these temperatures. As detection of this enzyme is important in current primary identification tests, the organism's potential for very rapid growth has not been exploited significantly in the diagnostic laboratory.

C. perfringens-like organisms, including *C. absonum*, *C. paraperfringens* and *C. sardiniensis*, differ in relatively minor characteristics from typical *C. perfringens* (14, 15). It is important to keep in touch with developments in this area.

Any approach that might help to sub-divide the *C. perfringens* complex merits exploration as our present dependence on animals for toxin neutralisation tests in the typing of the organism is a great disadvantage. The use of agglutination tests for the typing of strains involved in food-poisoning has been very helpful (10).

C. difficile. Our experience with this organism has recently been reviewed by Poxton (17). We characterise the organism on the basis of its microscopic morphology, its ability to grow on CCFA medium, its performance in biochemical tests, the characteristic profile of end-products detected by gas-liquid chromatography (GLC), and the detection of characteristic antigens in crossed immunoelectrophoresis systems when tested against rabbit antiserum raised against whole cells of the type strain (NCTC 11223). Antigenic cross-reactions with the *C. sordellii/bifermentans* complex can be

absorbed out. Some strains do not produce the typical cytotoxin and it is clear that more work is needed on the debated role of the cytotoxin in the enteropathogenicity of this organism. Some of our strains showed minor variations in the presently accepted range of biochemical tests used for characterisation; several showed significant differences, and two strains showed marked departures from the recognised pattern for *C. difficile* published in the VPI manual (12). Differences of this degree would certainly qualify for separate species rank in the *Bacteroides* genus (see below). We are anxious to determine whether the variation offers a basis for biotyping which may be of epidemiological value in tracing infective strains.

Neuraminidase production as an aid to clostridial taxonomy

The possible association of neuraminidase production with pathogenic potential is debated, but the enzyme may be of taxonomic use (8). Table I lists the neuraminidase-positive and neuraminidase-negative clostridia. This appears to distinguish clearly between *C. sordelli* and *C. bifermentans* and complements the case for regarding these as separate species.

Amino acid utilization and production in relation to taxonomy

Studies to explore the metabolism of amino acids by clostridia as an aid to our understanding of the proteolytic group deserve our attention (7).

Bacteroides-Fusobacterium group. Presumptive identification at genus level can be made by observations on Gram smears, colonial morphology and a few other tests. GLC analysis of short chain fatty acid end-products of metabolism can also identify to genus level, but further tests are required to establish the species or sub-species of *Bacteroides* and *Fusobacterium* (4). We select the observations and tests indicated in Table II as the currently recommended range (4, 5, 6, 11).

The experience of others (12, 13) encourages us to replace mannitol with salicin, and we note promising results with xylan (19). The range of organisms indicated in Table II is not complete and is intentionally restricted to those species that might merit specific recognition in the clinical laboratory. Dental bacteriologists may soon press for the inclusion of *Bacteroides gingivalis* as an important species. The restriction to genus level for *Fusobacterium* species is unwise and merely reflects our present lack of extensive experience with this group.

We now restrict the tolerance tests to taurocholate and to one dye only, in view of practical difficulties with Victoria blue. The two tests give information that helps significantly to compensate in a laboratory that might not be able to afford the expense of a GLC system.

Serological characterisation. In studies that exploit the species-specific antigenicity of the cell surface outer membrane (OM) complex and the sensitivity of an indirect enzyme-linked immunosorbent assay (ELISA), we are now able to identify many *Bacteroides* strains to species and sub-species level (16, 18). Table III summarises results obtained with OM preparations from representative reference laboratory and freshly isolated strains with species reference antisera that we prepared against whole cells and tested in an indirect ELISA system. The outer membrane preparations are obtained by treatment of bacterial suspensions with 10 mM EDTA at 45° C for 30 min and then holding in an ultrasonic bath for 60 s. The antisera are raised in rabbits against whole live bacterial suspensions. Heterologous cross-reacting antibody can be removed by absorption of the antiserum with whole cells. We find very significant correlation between serotype and biotype in this work. Results were clear-cut with the *B. fragilis* complex, the asaccharolytic group, and with *B. melaninogenicus intermedius*. The results with the *B. melaninogenicus-ruminicola* complex reflect the confusion that also afflicts conventional biotyping systems.

Table I. *Neuraminidase studies with clostridia*

Neuraminidase-positive clostridia	
C. perfringens, types A–E	
C. septicum	
C. chauvoei	
C. tertium	
C. sordelli	
C. absonum	
Neuraminidase-negative clostridia	
C. novyi, types A–D	*C. butyricum*
C. tetani	*C. sphenoides*
C. botulinum	*C. fallax*
C. sporogenes	*C. tetanomorphum=cochlearium*
C. bifermentans	*C. subterminale*
C. histolyticum	*C. difficile*
C. paraperfringens	

Table II. *A practical approach to the characterisation of commonly encountered gram-negative non-sporing anaerobic bacilli of clinical interest*

+ = > 95% of strains give positive result. − = > 95% of strains give negative result. v = variation 30–70%. +(−) = positive with most strains. −(+) = negative with most strains. For details see Duerden, Holbrook, Collee & Watt, 1976. J appl Bacteriol 40: 163–188. The characterization of clinically important gram-negative anaerobic bacilli by conventional bacteriological tests.

	B. fragilis	*B. vulgatus*	*B. distasonis*	*B. thio.*	*B. ovatus*	*B. mel. intermed.*	*B. mel. mel.*	*B. oralis*	*B. ruminicola*	*B. bivius*	*B. disiens*	*B. asaccharolyticus*	*B. ureolyticus*	*Fusobacterium* spp.[a]
Pigment	−	−	−	−	−	+	+	−	−	−	−	+	−	−
Indole	−	−	−	+	+	+	−	−	−	−	−	+(−)	−	v
Aesculin hydrolysis	+	v	+	+	+	−	v	+(−)	+	−	−	−	−	−(+)
Fermentation of														
Glucose	+	+	+	+	+	+	+	+	+	+	+	−	−	v
Lactose	+	+	+	+	+	−(+)	+	+	+	+	−	−	−	−(+)
Sucrose	+	+	+	+	+	+	+	+	+	−	−	−	−	−(+)
Rhamnose	−	+	+(−)	+	+	−	−(+)	v	+(−)	−	−	−	−	−
Trehalose	−	−	+	+	+	−	−	−	−	−	−	−	−	−(+)
Xylose	+(−)	+	+	+	+	−	−	−	+	−	−	−	−	−
Salicin	−	−(+)	+	−(+)	+	−	−	−(+)	+(−)	−	−	−	−	−(+)
Significant FA Produced[b]														
Succinic			++					++				(+)	+	−(+)
Acetic			++					++				++	+	+
N-Butyric			−					−				++	−	++
Tolerance of[c]														
Sod. Taurocholate			+					−				−	−	v
Gentian Violet			−					−				−	−	+
Resistance to[d]														
Neomycin 1000 µg			R					S				S/R	S	S
Kanamycin 1000 µg			R					R				R	S	S
Penicillin 2 units			R					S/R				S	S	S
Rifampcin 15 µg			S					S				S	S/R	S/R

[a] Alternative reactions in this column relate to species differences as well as strain variations.
[b] GLC: + = 1–10 µmol/ml. ++ = > 10 µmol/ml. (+) = variable amount of minor product.
[c] Tolerance: Taurocholate 0.5%. Gentian violet 1 in 100 000. + = tolerant. − = inhibited.
[d] Antibiotic resistance: R = resistant. S = sensitive. S/R = variation (30–70%).

Neuraminidase studies. Table IV lists the neuraminidase-negative and positive *Bacteroides* species and may help in classifying the *melaninogenicus-ruminicola-oralis* complex and the *bivius-disiens* group. The few *Fusobacterium* species that we have tested do not produce neuraminidase (9). Our neuraminidase tests helped to discriminate between closely related *Bacteroides* species that are presently differentiated by a small number of sugar fermentation reactions. As the results are consistent for all strains of each of the species that we examined, the findings might be of taxonomic value (9). In view of the technical difficulties of neuraminidase assays, it will be necessary to develop a simple test; for example, the enzyme's ability to confer panagglutinability on human red cells might be exploited.

Bacteriocin typing. Bacteriocin-like substances produced by *Bacteroides* species offer a possible approach to typing and sub-classification (22). It was first necessary to develop detailed characterisation studies and now it seems worthwhile to re-examine the "bacteroidicins" in which we were interested some years ago, but now to recognise them as fragilicins, etc. It has been possible to analyse our earlier results in the light of our subsequent characterisation of the organisms that we tested

Table III. *Results of serological studies with outer-membrane antigens and homologous antisera in an indirect ELISA system*

Species and strain(s) used as antigen	Autologous titre[a]	Number of homologous strains yielding preparations that reacted to at least 1 8 (out of number tested)
B. fragilis		
NCTC9344	12 800	34 (35)
B. vulgatus		
NCTC10583	25 600	11 (11)
B. distasonis		
ATCC8503	12 800[a]	
GNAB26	12 800	6 (8)
B. thio.		
NCTC10582	25 600[a]	
GNAB11	3 200	8 (10)
B. mel. intermed.		
NCTC9338	12 800	11 (11)
B. mel. mel.		
ATCC15930	12 800[a]	
VPI4196	25 600	6 (11)
B. oralis		
1210	51 200	5 (17)
B. ruminicola		
NP333	25 600[a]	
GA33	6 400	6 (14)
B. asaccharolyticus		
NCTC9337	25 600	24 (25)

[a] The autologous titre denotes the titre obtained with the strain used as antigen. When two antisera were prepared for a species, the combined results are given.

Table IV. *Results of neuraminidase studies with organisms of the Bacteroides-Fusobacterium group*

Neuraminidase-positive Bacteroides spp.	
B. fragilis	*B. mel. ss. melaninogenicus*
B. vulgatus	*B. mel. ss. levii*
B. distasonis	*B. oralis*
B. ovatus	*B. bivius*
B. thetaiotaomicron	
B. variabilis	
(B. ochraceus = Capnocytophaga)	
Neuraminidase-negative Bacteroides spp.	
B. eggerthii	*B. mel. ss. intermedius*
B. uniformis	*B. ruminicola*
B. splanchnicus	*B. disiens*
	B. asaccharolyticus
	B. corrodens
Neuraminidase-negative fusobacteria	
F. necrophorum	*L. buccalis*
F. polymorphum	
F. varium	
F. necrogenes	

(Table V). The indications are that most strains of *B. fragilis* are both good producers and indicators, whereas strains of *B. thetaiotaomicron* studied by us were poor in this respect. Our experience with the *B. melaninogenicus* group is presently inadequate. The results obtained with the *B. asaccharolyticus* strains seem to be promising, as the producers showed their effect only against *B. asaccharolyticus* indicators. These are retrospective analyses and a note of caution is needed: the agents responsible for these effects have not been formally characterised as bacteriocins and a systematic study is now indicated with a range of characterised strains.

The anaerobic cocci

We certainly confirm that "the clinical microbiologist attempting to isolate and characterise the obligately anaerobic cocci, and the clinician attempting to treat infections caused by these organisms, both suffer from a lack of useful data" (24). We accept the provisional definition of Watt and Jack (23) that anaerobic cocci grow under satisfactory conditions of anaerobiosis and do not grow on suitable solid media in 10% CO_2 in air even after incubation for 7 days at 37° C. These workers found that metronidazole sensitivity is a reliable index: obligately anaerobic cocci are all sensitive to metronidazole.

The detailed descriptions provided by Smith and Holdeman (20) provide a helpful starting point for a review of classification of this difficult group. We find the inclusion of *Streptococcus* confusing. The division of the streptococci into truly anaerobic and facultative groups might be taxonomically proper, but facultative strains are encountered in clinical specimens that cannot be isolated initially unless

Table V. *Results of preliminary studies of bacteriocin-like effects with gram negative anaerobic bacilli*

Species	Producers		Indicators	
	Number Positive	Number Tested	Number Positive	Number Tested
B. fragilis	34	37	28	34
B. thio.	1	6	1	6
B. vulgatus	0	5	4	4
B. distasonis	0	1	0	1
B. mel. ss. intermed.	2	2	0	2
B. mel. ss. mel.	1	1	1	1
B. asaccharolyticus	4	4	4	4
B. ruminicola	0	1	0	1
Fusobacterium spp.	2	5	0	3

anaerobic culture procedures are undertaken (20). This may be explained on the basis of carbon dioxide dependence and other exacting considerations that merit further work. Thus, apparently anaerobic cocci that are not true anaerobes still feature in our collections, and sometimes in our clinical reports. The anaerobic state and sensitivity to metronidazole is of clinical importance, but it is inevitable that the rules of convenience must bow to the stricter rules of a system that might well include obligate anaerobes and facultative organisms in one genus. Accordingly, the clinical bacteriologist must be on guard: we make the lighthearted but significant comment that some of our organisms at the primary isolation stage have not read the textbook.

The anaerobic spirochaetes

We are again indebted to Smith and Holdeman (20) for a helpful outline. In addition to the well-known human pathogenic associations of the anaerobic species in the genera *Spirochaeta*, *Borrelia* and *Treponema*, the association of spirochaetes with other organisms in periodontal disease is of much interest. The occurrence of spirochaetes in the human colon, especially noted in male homosexuals, also merits further study. Smith takes the view that it is doubtful whether any of the cultivable treponemes are significantly pathogenic in the mouth. The increase in number of these organisms in areas of gingival pathology has been recognised for years, but the correlation of these organisms with degrees of tissue destruction has stimulated interest among dental bacteriologists and dental practitioners concerned with periodontal disease (21). There is pathogenic synergy in these infections and, while it may be that the spirochaetes are not a significant component of the initial microbial attack and may be merely secondary opportunists exploiting conditions that encourage their growth, many of us have been slow to develop adequate investigations of periodontal disease. It is likely that the significant primary pathogens will include bacteroides organisms and fusobacteria and perhaps *Capnocytophaga*-like organisms which abound in these lesions. Recent developments in the classification and characterisation of these organisms should now allow us to make significant progress in this important field.

CONCLUSION

Our aim in this paper has been to consider the degrees of precision regarded as necessary and practical in the characterisation and classification of some representative anaerobic bacteria of clinical importance. It is paradoxical that the label of *C. perfringens* presently applies to a remarkably wide range of human and animal pathogens that can only be distinguished by difficult toxigenic analyses, whilst new species rank is afforded to relatively minor variant biotypes. In this case, serological analyses of soluble antigens have so far helped only in typing food-poisoning strains. On the other hand, *C. difficile* seems to embrace many biotypes: present confusion concerning the significance of the cytotoxin and the enterotoxin in its identification encourages us to develop somatic serological studies. Further studies of amino acid metabolism might also be helpful. The successful biotyping of the *Bacteroides-Fusobacterium* group has been extended to the point at which minor biochemical variants qualify for species rank, and this seems to be supported by serological analyses of species-specific outer membrane antigens. Other possible approaches include bacteriocin typing and this may have epidemiological value. In contrast, the classification of the anaerobic cocci is still poorly developed, and the oral spirochaetes merit more systematic study.

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Bacteroides, Fusobacterium and related organisms: anaerobic cocci: identification of anaerobes

This chapter deals with the *Bacteroides/Fusobacterium* group of organisms and, for practical reasons, it embraces *Mobiluncus* and the anaerobic cocci. Many of the principles adopted for laboratory diagnosis and identification of these organisms are also applicable to the clostridia, which are covered in the next two chapters. Some procedures basic to anaerobic technology, such as anaerobic culture methodology, have been detailed in earlier sections of this book (Ch. 7). The first part of this chapter outlines a general approach to the identification of anaerobic bacteria, relevant not only to this chapter but also to the succeeding chapters on clostridia.

General approach to the identification of anaerobic bacteria

Table 36.1 at the end of this chapter outlines the range of tests and media that are of value in characterizing the different groups of strictly anaerobic Gram-positive and Gram-negative bacteria. As it is easy to presume that a demanding aerobe or facultative anaerobe might be an obligate anaerobe, it is most important to subject isolates to a systematic screening procedure as indicated in the first few steps of Table 36.1.

Cell morphology and Gram-stain reaction should be examined in films from blood agar (BA) or cooked meat broth (CMB) cultures incubated for 18–24 h, or longer for anaerobes that grow slowly. Similarly, colonial morphology on BA plates may be examined after 24 h, but prolonged incubation is required for slow growers.

Some organisms, e.g. *Bacteroides melaninogenicus*, produce dark brown or black colonies on blood-containing media, by forming a haemprotein complex. Up to 14 days incubation may be required before this is seen; pigment formation is less delayed if lysed blood is used. Fluorescence of colonies can be detected by exposing BA cultures to long-wave UV light (365 nm) from a Wood's lamp. Colonies of *B. melaninogenicus* fluoresce brick-red, and *Clostridium difficile* colonies fluoresce green. Colonies of fusobacteria fluoresce yellow on media containing cysteine hydrochloride. Note that other clostridia may also fluoresce and that *B. asaccharolyticus* and *B. bivius* colonies on blood agar are pink, red, dark red and purple under UV light at successive stages of growth over 1–4 days.

The further tests indicated in Table 36.1 extend into a range of biochemical and resistance tests that may be of value for determining the individual species within each of the broad groups of organisms. Practical experience with the characterization steps indicated in the subsequent tables in this chapter (or Ch. 37 for clostridia) will allow a bacteriologist to discriminate in the number of tests used for a particular identification, e.g. depending on the nature of a Gram-negative rod isolated, a short-cut approach along the lines set out in Table 36.2 at the end of this chapter might be adopted.

Detection and quantitation of fatty acid end-products of bacterial metabolism by *gas chromatography* (GC) is a useful aid to the identification of anaerobic bacteria. When this information is used in conjunction with details of cell morphology and Gram-stain reaction, it

is possible to establish the identity of anaerobes to genus level (as in Table 36.3), and occasionally to species level. The use of GC in characterization of Gram-negative anaerobes is included in the further tables in this chapter, and accounts of GC for identification of the clostridia are given in Chapters 37 and 38. Technical details are given in specialist manuals (or see Deacon et al 1978); a brief account is provided in the *Methods* section of this chapter.

BACTEROIDACEAE

The strictly anaerobic Gram-negative non-sporing rod-shaped bacteria are abundantly represented as commensals in the human gastrointestinal tract, oropharynx and female genital tract and these include many opportunist pathogens. It is increasingly evident that these anaerobes are involved in a very wide range of clinical conditions and that characterization of individual species concerned can be of clinical significance. This section deals with the *Bacteroides*, *Fusobacterium* and *Leptotrichia* organisms of clinical interest. The description and laboratory guidelines are closely linked with the information given in the associated tables (see end of chapter). Many laboratories find it necessary to restrict the degree of characterization. The information given in Table 36.2 illustrates a short-cut approach in this complex field.

Morphology

Range from short Gram-negative rods to filamentous and fusiform shapes. Pleomorphism is common. All are non-sporing, but some may produce swollen spherical bodies resembling sporing forms when wet films are examined by phase contrast microscopy. Most species of clinical interest are non-motile.

Cultural characters

The nutritional requirements of this heterogeneous group vary. Most of the species of medical interest grow better on enriched media such as freshly prepared or pre-reduced blood agar. Development of visible colonies of some strains may require 48 h or more. Some have special requirements for haemin and vitamin K3 (menadione). The presence of 10% CO_2 in the anaerobic atmosphere often markedly enhances growth. Colonial appearances vary, depending on species and culture media, from tiny translucent colonies to large, grey, circular or irregular colonies at 24–48 h.

Biochemical activities

Active saccharolytic and some non-saccharolytic species are described. Some are proteolytic and a few produce other demonstrable exoenzymes that facilitate their early characterization (see below).

Sensitivity to physical and chemical agents

These organisms vary in their sensitivity to oxygen; in general, colonies on solid media should be picked promptly for subculture. Species variations in sensitivity to dyes, bile and antibiotics can be exploited in simple characterization systems (Table 36.2).

Antibiotic sensitivity

In general, organisms of the *B. fragilis* group are resistant to penicillin and produce potent cephalosporinases and penicillinases. A significant proportion of organisms in the *B. melaninogenicus* group are partially or markedly resistant to penicillin. The fusobacteria are generally sensitive to penicillin. All of these anaerobes are resistant to clinically achievable concentrations of the aminoglycosides. They are often resistant to the tetracyclines. They are generally sensitive to clindamycin, and virtually always sensitive to chloramphenicol and metronidazole.

Serological identification

Several different attempts pioneered by Beerens et al (1971) to develop a serological scheme for identifying *Bacteroides* spp. have been largely

empirically based on heated or unheated whole cell antigens and procedures such as agglutination or immunofluorescence. In 1987, when this chapter was written, no serological scheme was widely in use. Two immunofluorescence kits available commercially (Fluoretec F and Fluoretec M; Pfizer) for the identification of the *B. fragilis* group and *B. melaninogenicus* spp. do not differentiate between the likely pathogens and non-pathogens and are still being evaluated. The F kit seems to react with the majority of *B. fragilis* strains but gives variable results with other species in the *B. fragilis* group. The M kit reacts with some *B. bivius* and *B. melaninogenicus* strains, but reactions within this group are not sufficiently specific. As more is understood of the antigens of *Bacteroides* spp., more specific serological schemes will be developed for the identification of these organisms. At present, capsular antigens and outer membrane protein antigens both appear promising for exploitation. The highly heterogeneous LPS antigens have been used in exploratory epidemiological studies.

Other typing methods

Promising results are being obtained with bacteriocin typing, which is simpler than serotyping and may be of diagnostic and epidemiological value (Riley & Mee 1982).

Animal pathogenicity

Animal pathogenicity tests are not performed routinely for any Gram-negative anaerobe. In our limited experience with fusobacteria, *F. necrophorum* is lethal for rabbits when injected intravenously. Various animal models have demonstrated that pathogenic synergy exists between some facultative anaerobes such as *Escherichia coli* and *Bacteroides* spp. (Onderdonk et al 1977; Kelly 1978).

Classification of Bacteroidaceae

The Gram-negative anaerobic non-sporing bacilli of clinical interest are essentially contained within the *Bacteroides/Fusobacterium* group (Bacteroidaceae). A spectrum of Gram-negative rods in this range of organisms extends across many genera, linked by unnamed and, as yet, inadequately characterized groups. There are associated non-anaerobic genera to add to the confusion; recently described genera with characterized species of medical and particular dental interest include the *Eikenella* group which is aerobic and includes capnophilic members, and *Wolinella* which is a group of anaerobic or microaerophilic species. The microaerophilic capnophilic organism formerly known as *Bacteroides ochraceus* is now in the genus *Capnocytophaga* (*C. ochracea*). These 'associated non-anaerobic genera' are not discussed further in this chapter, but there is a note on *Mobiluncus*. *Gardnerella* is described in Chapter 19.

By 1987, there were more than 30 species of *Bacteroides* and 14 species of *Fusobacterium*. Many species occur as normal commensals in man, but some have much greater pathogenic potential than others. *B. fragilis* is accepted as the most commonly encountered pathogenic member of the genus, but other pathogenic associations are increasingly recognized.

Several schemes have been developed for the identification of Gram-negative anaerobic rod-shaped bacteria. Those most widely used are based on publications of the Virginia Polytechnic Institute (Holdeman et al 1977), the Center for Disease Control, Atlanta (Dowell & Hawkins 1976) and the Wadsworth Hospital, Los Angeles (Sutter et al 1975). A shortened, simple and reliable scheme is that of Duerden et al (1980). *Bacteroides* and *Fusobacterium* can be readily separated into major groups by simple tests (Table 36.2) and these groups can be subdivided to species level by further biochemical tests (Tables 36.4–36.7; all at the end of this chapter). The *Bacteroides* organisms can be divided into the *B. fragilis* group, the *B. melaninogenicus/oralis* group, and an asaccharolytic group. In GC tests, the fusobacteria characteristically produce major amounts of butyric acid with varying amounts of other fatty acids but generally excluding iso-butyric and iso-valeric acids. On the other hand, most *Bacteroides* organisms produce major amounts of acetic and succinic acid; the few species that produce butyric acid usually also

produce iso-butyric or iso-valeric acid or both of these (see Table 36.3).

Note. In the tables that give differential characteristics of important pathogens in this group of organisms, key reactions are indicated in bold and these provide a short-cut characterization procedure of value to hard-pressed clinical laboratories.

Bacteroides fragilis group

This comprises non-pigmented, strongly saccharolytic and bile tolerant species of *Bacteroides* and includes *B. fragilis*, *B. vulgatus*, *B. distasonis*, *B. thetaiotaomicron*, *B. ovatus*, *B. uniformis*, *B. variabilis*, *B. eggerthi* and *B. splanchnicus* (see Table 36.4). They are non-motile, non-sporing, small Gram-negative rods 1–4 × 0.4–0.8 μm with rounded ends. Pleomorphism is common and large bizarre rods with round or oval swellings can be seen if fermentable carbohydrate is present. They grow well on freshly prepared blood agar incubated anaerobically with 10% CO_2 at 37°C to produce smooth, circular, convex, opaque, light grey colonies 1–2 mm in diameter at 24 h that are usually non-haemolytic. All members of this group are resistant to penicillin.

Bacteroides fragilis. This organism has the characteristics of the group described above. It is not the most numerous of the commensal bacteroides but it is the most commonly encountered member of the group in a pathogenic role, occurring in postoperative infections associated with abdominal and gynaecological surgery, and in a wide range of other infective situations including cerebral abscess, lung abscess and soft tissue infections, often in concert with other pathogens. The infections are usually endogenous, often assisted by debilitating factors that operate locally or generally in the patient.

It is thought that *B. fragilis* owes its special pathogenic potential to its production of capsular material, but the factor or factors primarily related to the virulence of this organism are still debated. In common with some other pathogenic bacteroides organisms, *B. fragilis* can inhibit the effective phagocytosis and killing of coliform organisms in test mixtures with polymorphs; this is a likely explanation of the pathogenic synergy exhibited in mixed infections with these organisms. Aggressins produced by *B. fragilis* that may contribute to virulence include a range of proteases, neuraminidase, DNase, heparinase and other enzymes. In addition to this range of biochemical activity against complex substrates, the organism has other activities including the ability to hydrolyse aesculin and to ferment several sugars actively; the latter characteristics are of use in sub-classification (see Table 36.4). *B. fragilis* produces very potent β-lactamases and these preclude the use of antibiotics of the penicillin series and most of the cephalosporin drugs, though cefoxitin has some activity. Some strains are resistant to the lincomycins. All but a very few strains are sensitive to metronidazole which is most often the drug of choice.

Bacteroides melaninogenicus/oralis group

This group (Table 36.5) comprises saccharolytic, bile-sensitive species of *Bacteroides*, some of which produce black-pigmented or brown colonies on blood-containing media. The pigmented species *B. melaninogenicus*, *B. intermedius*, *B. corporis* and related organisms not specified here are non-motile, non-sporing, small Gram-negative rods, 0.6–2 × 0.4 μm, often coccobacillary, and sometimes pleomorphic in broth cultures. They grow well on freshly prepared blood agar supplemented with menadione 1 μg/ml incubated anaerobically plus 10% CO_2 at 37°C. At 24 h colonies may be very small; at 48 h they are 1 mm, smooth, circular, convex and opaque. The colour of the colony changes from light grey, through tan and brown, to black. Speed of pigment development is enhanced on lysed blood agar, or by spotting a haemolytic organism on to an inoculated blood agar plate before incubation. Detectable development of pigment by some species may take up to 7 days or more. Colonies of *B. melaninogenicus* on blood agar, and cells of the species in pus or on wound swabs, will fluoresce brick-red when subjected to long-wave (365 nm) UV light (Myers et al 1969).

Non-pigmented members of this group include *B. oralis*, *B. ruminicola*, *B. bivius* and *B. disiens*. Several new species have recently been proposed.

The cellular and colonial morphology is similar to that of the pigmented members, except that cells are often slightly larger and colonies are light grey to light tan and do not fluoresce brick-red.

B. melaninogenicus and related organisms are associated with periodontal disease, gingivitis, dental abscesses, maxillo-facial sepsis, lung abscess and cerebral abscess. The infections are often mixed. In acute ulcerative gingivitis (Vincent's infection) *Bacteroides* organisms occur in pathogenic synergy with Vincent's spirochaete and a fusiform organism which may be *Fusobacterium nucleatum* or *Leptotrichia buccalis*. The appearances in a Gram smear are diagnostic. Pigmented and non-pigmented members of the *melaninogenicus/oralis* complex are associated with infections of the female genito-urinary tract. These are assumed to be endogenous as the organisms are encountered frequently (but not invariably) as components of the commensal flora of that site. However, *B. fragilis* is still regarded as the commonest Gram-negative anaerobic pathogen in female genital tract infections (see Duerden 1980, 1984).

Asaccharolytic bacteroides group

This group (Table 36.6) comprises non-saccharolytic or weakly saccharolytic, usually bile sensitive, species of bacteroides, some of which produce black pigmented colonies on blood-containing media. The pigmented species, *B. asaccharolyticus*, *B. endontalis* and *B. gingivalis*, are non-motile, non-sporing, small Gram-negative rods, 1 × 0.6 μm, often coccobacillary and pleomorphic. Colonies on freshly prepared blood agar are similar to pigmented members of the *melaninogenicus/oralis* group.

The non-pigmented asaccharolytic members include a number of species, some of debatable status. Cell and colonial morphology vary among the species. *B. praeacutus* is motile, and colonies of *B. ureolyticus* burrow into the surface of agar plates.

Fusobacteria and Leptotrichia

Representative species of fusobacteria are listed in Table 36.7.

These organisms tend to occur in mixed infections such as putrefactive necrotic fusospirochaetal conditions with much tissue destruction, as in cancrum oris, other maxillo-facial infections (sometimes following surgical excision for neoplastic disease), septic abortion and lung abscess. Sometimes a fusobacterial infection can be fulminating and can advance with great rapidity from a sore throat syndrome to a necrotizing pneumonitis. Fusobacteria are commonly involved in dental and periodontal infections, ulcerative gingivitis (see above), liver abscess and cerebral abscess. Fusobacteria and bacteroides organisms are often involved in concert or separately in necrotizing conditions variously described as anaerobic cellulitis, necrotizing fasciitis and dermal gangrene. The occurrence of these organisms in mixed infections with foul pus is common in infected pilonidal cysts, perianal abscesses, balanitis, and ulcers of the leg and buttocks, including diabetic ulcers and bed sores. Some species of fusobacteria produce long slender Gram-negative rods that are wide at the centre and taper towards the ends (fusiform), but others produce cells that range from coccobacilli to long slender rods with parallel sides and these are indistinguishable from other members of the Bacteroidaceae. Isolation is best on blood agar plates containing neomycin and vancomycin.* They are strict anaerobes, sensitive to metronidazole, and tolerant of gentian violet. Most are non-motile. After incubation at 37°C for 48 h colonies usually have a raised, irregular or crenated edge, a peaked centre, and may be striate and granular.

Fusobacterium nucleatum is the type species of the genus. It is a slender spindle-shaped Gram-negative rod, 5–10 × 1 μm, with tapering pointed ends and often with central or eccentric swellings. After incubation for 48 h on freshly prepared blood agar, colonies are 1–2 mm, irregular, low convex, translucent or opaque, grey and non-haemolytic. The organism is weakly saccharolytic, non-proteolytic and non-motile.

Fusobacterium necrophorum. This Gram-negative anaerobe has a tendency to pleomorphism and irregular staining. Cells are 1–10 × 0.5 μm; long filaments are frequently

* Refer to *Methods* at end of this chapter.

seen. They are non-motile and non-sporing, but swollen areas sometimes occur. At 48 h, colonies are 1–2 mm, circular, with a flattened edge and raised or convex centre, translucent or opaque, grey and often β-haemolytic. Most strains are lipolytic (see *Methods*, Ch. 37).

Leptotrichia buccalis. This organism is characteristically seen in Gram smears of clinical specimens as long Gram-negative rods of considerable width and with terminal tapering; individual cells may be up to 15 × 1.5 μm. It is sensitive to metronidazole and tolerant of gentian violet. Colonies on blood agar after incubation for 48 h are 2–3 mm, very irregular and often striate. *Leptotrichia* has been thought of as essentially commensal in the human mouth, but pathogenic associations with other bacteria in the oropharynx are likely to be accepted.

Laboratory diagnosis of bacteroides and fusobacterial infections

The specimens should ideally be of pus, wound exudate, tissue or blood which has been transported to the laboratory in a closed syringe or in a container that has been flushed out with nitrogen. Swabs are not ideal; if they must be used, they should be inoculated on to the media directly, or transported to the laboratory in a reduced transport medium, or thrust into a semi-solid agar medium to avoid desiccation in transit. On receipt, specimens must be processed without delay. Clinical specimens containing fusobacteria or bacteroides can be handled briefly on the open bench and surface plating is suitable, provided that it is expeditious. An anaerobic cabinet (Ch. 7) is not essential, but can be useful if single specimens arrive sporadically or if cultures are to be checked regularly. Freshly poured plates or pre-reduced plates should be used. When seeded with a suitable inoculum, media should be promptly placed in an anaerobic environment with 10% CO_2 and incubated. The steps in a routine investigation are as follows:

1. Make a direct examination of the specimen. Note if it has a foul smell. Observe under a long-wave (365 nm) UV lamp for brick-red fluorescence, which suggests the presence of *B. melaninogenicus*. Consider preparing an ether extract of the specimen for direct examination by gas chromatography* (Holdeman et al 1977).

2. Prepare films for phase contrast microscopy and Gram stain. Observe characteristic morphology: non-motile, pleomorphic, coccobacillary to fusiform or filamentous, Gram-negative. Consider fluorescent antibody stains for bacteroides, but note cautions (see above).

3. Plate on to two plates each of blood agar (Columbia base containing 4 or 8% whole blood and 1 or 2% freeze-thawed lysed blood and menadione 1 μg/ml*) and two selective blood agar plates*. Primary sensitivity tests can be made at this stage by placing disks, e.g. metronidazole 5 μg and penicillin 1 or 2 units, on the first series of streaks. Also seed a tube of pre-reduced cooked meat broth (CMB).

4. Incubate one blood agar plate aerobically and the rest anaerobically with 10% CO_2 at 37°C. Check the CMB, the blood agar and one selective plate after 24 h. Leave the remaining selective plate undisturbed for 48 h.

5. Examine plates for typical colonies. Examine CMB enrichment by microscopy, and subculture as in (3).

6. Pick colonies and subculture into pre-reduced CMB, into PPY medium for subsequent identification by biochemical tests, and into PPY medium containing glucose 1% for gas chromatography,* and also to observe whether or not glucose is fermented.

7. Identification to group level is by observing the tolerance of the organism to bile and gentian violet,* its resistance to a range of antibiotics* and its ability to ferment glucose. Full identification to species level is by selecting the appropriate biochemical tests as indicated in Tables 36.1–7. A more detailed approach is described by Bennett & Duerden (1985).

MOBILUNCUS SPECIES

These are anaerobic or microaerophilic, curved, Gram-negative or Gram-variable rods and they are motile with two or more subterminal flagella. *Mobiluncus* spp. have antibiotic sensitivity profiles that are more typical of Gram-positive

organisms, and some features of their cell wall structure are more typical of Gram-positive cells, but this is debated. The organisms may occur in the vagina of apparently healthy women, but seem to have an association with non-specific or bacterial vaginosis. The organisms are fastidious and grow slowly at 33–37°C, producing round, entire, convex, smooth, translucent, colourless colonies, 2–3 mm in diameter, after anaerobic incubation on blood agar medium for 5 days. A suitable selective medium is blood agar supplemented with colistin 10 μg/ml and nalidixic acid 10 μg/ml. Two species are currently recognized, *M. curtisi* and *M. mulieris*. Both accepted species are saccharolytic, but can be differentiated as follows:

M. curtisi is 1.7 × 0.5 μm, Gram-variable, metronidazole resistant, and gives positive results in tests for hippurate and arginine hydrolysis and ONPG. Small amounts of acetic acid and major amounts of succinic acid are produced in glycogen-containing media.

M. mulieris is 2.9 × 0.5 μm, Gram-negative, metronidazole sensitive, and gives negative reactions in tests for hippurate and arginine hydrolysis and ONPG. Major amounts of acetic and succinic acids, and sometimes small amounts of lactic acid are produced in glycogen-containing media.

ANAEROBIC COCCI

Strictly anaerobic Gram-positive cocci have been assigned to the genera *Peptococcus, Peptostreptococcus, Ruminococcus* and *Sarcina*; strictly anaerobic Gram-negative cocci are included in *Veillonella, Megasphera* and *Acidaminococcus*. This brief account is restricted to *Peptococcus, Peptostreptococcus* and *Veillonella*, as these are the genera most commonly associated with clinical infections. Most of the peptococci have now been reclassified as peptostreptococci. *Peptococcus niger* is the only surviving member of the genus *Peptococcus* (see Table 36.8 and Ezaki et al, 1983).

Many species of anaerobic cocci are commonly found as commensals on the human skin, in the female genital tract, in the oropharynx, and in the gastrointestinal tract. These commensal organisms may contaminate clinical specimens. Anaerobic cocci may nevertheless be significant pathogens in intra-abdominal infections, brain abscesses, empyema and aspiration pneumonias, hepatic abscesses, infections of the female genital tract, or infections following maxillofacial surgery in debilitated patients. An association of anaerobic cocci with infected sebaceous cysts is recognized, and anaerobic cocci may also occur in many mixed infections of skin and soft tissues.

The anaerobic cocci are generally sensitive to metronidazole and penicillin, and to a wide range of other antimicrobial drugs including tetracycline, erythromycin, chloramphenicol, clindamycin and the cephalosporins. However, variations in sensitivity make it necessary to check the susceptibilities of isolates in the laboratory. Some cocci that may seem to be anaerobic on primary isolation but are resistant to metronidazole can be shown on subculture to grow aerobically, or microaerophically, or in a CO_2-enriched atmosphere; these isolates are not true anaerobic cocci (Watt & Jack 1977).

Laboratory diagnosis of anaerobic cocci

Table 36.8 indicates some of the representative species and a range of useful tests. Metronidazole sensitivity is used to differentiate true anaerobic cocci from those that may seem to be anaerobic on primary isolation. Vancomycin resistance is typical of Gram-negative anaerobic cocci. The peptococci and some species of peptostreptococci are resistant to novobiocin. Most strains of *Peptostreptococcus anaerobius* are sensitive to sodium polyanetholsulphonate (Liquoid), giving inhibition zones of more than 12 mm diameter with disks containing 100 μg Liquoid/disk; most other anaerobic cocci are resistant in this test. The species noted below merit special attention.

Peptostreptococcus anaerobius. Spherical or lanceolate Gram-positive cocci, diameter 0.8 μm. Short chains with some single and paired cocci are seen in smears from broth culture. Abundant gas produced in broth culture. Colonies on blood agar after anaerobic incubation with 10% CO_2

for 24 h at 37°C are 0.5–1.5 mm in diameter, round, convex, shiny, opaque, grey and non-haemolytic. After further incubation, colonies may become slightly irregular in outline, umbonate, with a light grey centre and darker grey periphery.

Peptostreptococcus magnus. Spherical Gram-positive cocci, diameter 0.8–1 μm. Small and large irregular masses with some single and paired cocci are seen in smears from broth culture. Slight gas production in broth culture. Colonies on blood agar after incubation for 24 h are very small, round, convex, shiny, opaque, grey and non-haemolytic.

Peptostreptococcus asaccharolyticus is similar to *P. magnus*, but colonies on blood agar after incubation for 24 h are usually 0.8–1 mm in diameter. Despite its name, it produces abundant gas in broth culture.

Veillonella parvula. Spherical Gram-negative cocci, diameter 0.3–0.5 μm. Diplococci and single cocci with some clusters are seen in smears from broth culture. Abundant gas is produced in broth culture. Colonies on blood agar after incubation for 24 h are very small, round, convex, shiny, opaque, light grey and non-haemolytic.

METHODS

Growth factors for anaerobes

The inclusion of reducing substances (cooked meat particles, cysteine hydrochloride, sodium thioglycollate, glucose) in culture media improves the growth of anaerobic bacteria. Some anaerobes have a requirement for other growth factors that are not present in the basal media; the addition of haemin and menadione (vitamin K3) enhances the growth of many species of bacteroides. There are claims that the growth of anaerobic cocci is enhanced by Tween 80 (Holdeman et al 1977); the authors could not demonstrate this effect in PPY medium.

Haemin and menadione

Haemin 500 μg/ml. Dissolve 50 mg haematin hydrochloride in 1 ml of 1 mol/litre NaOH solution, and make up to 100 ml with distilled water. Filter sterilize.

Menadione 100 μg/ml. Dissolve 10 mg menadione in 2 ml ethanol, and make up to 100 ml with distilled water. Filter sterilize. Protect from light.

Equal volumes of haemin and menadione solutions can conveniently be mixed before storage. They are added to all media for growth of bacteroides, but can safely be added to all media for growth of anaerobes with no apparent inhibitory effect. The final concentrations in the medium should be haemin 5 μg/ml and menadione 1 μg/ml.

Cysteine HCl 3.75%

Dissolve 3.75 g L-cysteine hydrochloride in 100 ml distilled water. Autoclave at 121°C for 20 min. The final concentration in the medium should be 0.075%.

Sodium carbonate 2%

Dissolve 2 g Na_2CO_3 in 100 ml distilled water. Filter sterilize. The final concentration in the medium should be 0.04%.

Tween 80 10%

Mix 10 ml Tween 80 and 90 ml distilled water. Autoclave at 121°C for 20 min. The final concentration in the medium should be 0.02–0.1%.

Media for anaerobes

Many media in general use are given in Chapter 6. Here we list several media that are specially modified for anaerobic work.

Thioglycollate medium

For fermentation and urease tests use Thioglycollate medium, without glucose or indicator (BBL), supplemented with 0.25% Yeast extract (Oxoid) and 0.25% sodium succinate. Dispense in 5 ml volumes and autoclave at 121°C for 20 min.

Proteose peptone–yeast extract broth (PPY)

Proteose peptone (Oxoid)	2%
Yeast extract (Difco)	1%
NaCl	0.5%

Dissolve the ingredients in distilled water and adjust the pH to 7.4. Dispense in 5 ml volumes and autoclave at 121°C for 20 min. For use, drive off dissolved oxygen by placing in boiling bath for 10 min, then add:

L-cysteine HCl, sterile 3.75% solution	0.1 ml
Na_2CO_3, sterile 2% solution	0.1 ml

Note: PPY medium with glucose 1% (PPYG medium is used for gas chromatography (see below).

Blood agar for anaerobes

The basic medium is Columbia agar (Oxoid). It is autoclaved at 121°C for 20 min and cooled to about 50°C, when the following are added to give:

Blood agar: 5% defibrinated horse blood.

Lysed blood agar: 5% saponin-lysed horse blood, and menadione to 1 μg/ml.

Combined blood/lysed blood agar: 4% whole blood, 1% freeze-thawed lysed blood and 1 μg/ml menadione. (Use of this medium allows haemolysis to be detected, and speeds up the formation of pigment by pigmented species of bacteroides.)

Selective blood agar

A range of agents can be added to any of the above blood agar media to make them selective for anaerobes as follows (the concentrations given are the final concentrations in the complete medium):

Selective medium for anaerobes. Add gentamicin 20 μg/ml.

Selective medium for bacteroides. Add kanamycin 75 μg/ml and vancomycin 7.5 μg/ml.

Selective medium for fusobacteria. Add neomycin 100 μg/ml and vancomycin 7.5 μg/ml.

Selective medium for clostridia. Add neomycin 70 μg/ml.

Fermentation tests for anaerobes

Dissolve the carbohydrates in distilled water and filter sterilize. Rhamnose, trehalose, melibiose, aesculin, xylan, cellobiose, inositol and mannose are prepared as 10% solutions, and used at a final concentration of 0.5%; the other substrates are prepared as 20% solutions, and used at a final concentration of 1%. Add 0.25 ml amounts of the appropriate test carbohydrate solutions to 5 ml amounts of reduced thioglycollate medium or PPY broth.

After inoculating the test organism, incubate anaerobically with 10% CO_2 at 37°C for 24 h, or up to 4 days until reasonable growth occurs. A seeded tube of the test medium without carbohydrate is incubated as a reference control. Read the results, preferably with a pH meter, or by adding a suitable pH indicator solution (e.g. a 0.02 ml drop of a 0.1% aqueous bromothymol blue solution). If a pH meter is used, the test is regarded as positive if the pH falls 0.5 unit below that of the carbohydrate-free reference control culture. If the indicator is used, a yellow colour indicates a positive result, and a green colour indicates a negative result. It is not advisable to incorporate an indicator in the culture medium at the start as indicator dyes may be decolourized, sometimes irreversibly, under anaerobic conditions. When equivocal results are obtained with the indicator solution, they should be verified with a pH meter.

Other tests modified for anaerobes

Urease

Dissolve the urea (10%) in distilled water and filter sterilize. Perform this test in thioglycollate medium or proteose peptone yeast extract (PPY) broth containing 1% urea, and read the results as for the fermentation tests. The test is regarded as positive if the pH rises 0.5 unit above that of the substrate-free reference control culture, or if the indicator turns blue.

Indole production

Extract 5 ml of PPY culture with 0.5 ml toluene. Slowly layer 0.5 ml Ehrlich's indole reagent on

to the liquid interface. A red/purple colour indicates a positive result.

Ehrlich's indole reagent. Dissolve 1 g *p*-dimethyl-aminobenzaldehyde in 95 ml absolute ethanol, then slowly add 20 ml concentrated HCl. Protect from light.

Nitrate reduction

Sterilize a solution of potassium nitrate (KNO_3) 2% in distilled water by autoclaving at 121°C for 20 min. Use this to supplement PPY medium with KNO_3 200 μg/ml. To 5 ml of the test culture in this PPY nitrate medium, add 0.25 ml Reagent 1, then 0.25 ml Reagent 2. A red colour indicates the presence of nitrites.

Reagent 1. Add 100 ml distilled water and 30 ml glacial acetic acid to 0.5 g sulphanilic acid and allow to dissolve.

Reagent 2. Dissolve 0.2 g Cleves acid (5-amino-2 naphthalene sulphonic acid) in 120 ml distilled water by warming in a waterbath, then add 30 ml glacial acetic acid.

Gas chromatography (GC) of metabolic products

This procedure can be used with cultures of test organisms, or directly with pus or exudate. Grow cultures for 24 h or longer in 5 ml of PPY medium containing 1% (w/v) glucose (PPYG). After acidification to *c.* pH 2 with 0.1 ml of 50% (v/v) H_2SO_4, remove the bacteria and insoluble material by centrifugation at 5000 *g* for 15 min. For analysis of volatile fatty acids (VFA) and alcohols, prepare ether extracts by adding 0.2 ml 50% (v/v) H_2SO_4, 0.4 g NaCl and 1 ml diethyl ether to 1 ml of the prepared culture supernate. After mixing well and centrifuging at 500 *g* for 5 min, carefully pipette the ether layer on to fine-mesh anhydrous $CaCl_2$ to remove water. The $CaCl_2$ should be about one quarter of the total volume, and the mixture should be left for 5 min before use (Holdeman et al 1977). Inject samples (1 μl) into the GC column. It is possible to obtain VFA profiles by injecting samples (1 μl) of acidified culture supernate directly on to some GC columns (Deacon et al 1978) but these columns become contaminated and the packing must be replaced. Ether extraction is essential for samples of pus or exudate. For non-volatile fatty acids (lactic, succinic and phenylacetic), methylate samples by mixing 1 ml of prepared culture supernate, 0.4 ml of 50% (v/v) H_2SO_4 and 2 ml methanol in a stoppered tube and either leave overnight at room temperature, or heat at 60°C for 1 h. Add 1 ml distilled water and 0.5 ml of chloroform and mix well by inversion. If an emulsion forms, centrifuge at 500 *g* for 5 min. Inject samples (1 μl) of the chloroform layer on to the GC column.

A less hazardous solvent, methyl *tert*-butyl ether, can replace both diethyl ether and chloroform for the extraction of volatile and non-volatile fatty acids (Thomann & Hill 1986).

Following separation of the acids in the column, they are detected and then recorded as a series of peaks on a chart recorder. Identification and quantitation of the substances producing these peaks is done by comparing the retention time and the peak area respectively with peaks produced by standard amounts of known acids.

Useful column packings for analysing metabolic products include; FFAP, SP 1220 (15%) + H_3PO_4 (1%), or SP 1000 (10%) + H_3PO_4 (1%) on 100/200 Chromosorb WAW (Supelco), or Chromosorb 101, 100/120 mesh (Supelco, or Phase Sep).

Aesculin hydrolysis

Aesculin cooked meat broth is prepared similarly to CMB, except that aesculin is dissolved in the broth component before autoclaving. Add 0.5 ml of a 1% aqueous ferric ammonium citrate solution to the test culture in CMB containing 1% aesculin, or PPY containing 0.2% aesculin. A black colour indicates a positive result. *Note*: renew the ferric ammonium citrate solution when it changes from green to brown.

Gelatin digestion

Observe a charcoal gelatin disk incubated in a CMB culture of the test organism for up to 2

weeks. Release of charcoal indicates digestion of the gelatin.

Prepare disks by a modification of Kohn's method (1953). Dissolve 12.5 g gelatin (Difco) in 100 ml nutrient broth (Oxoid No. 2). Add 5 g of finely powdered charcoal, pour the mixture into flat dishes to a depth of *c.* 3 mm and allow to solidify at 4°C. Hold the charcoal gelatin in 4% aqueous formaldehyde solution at room temperature for 5 days and then, after rinsing briefly in tap water, cut 1 cm disks. Leave the disks in running tap water for 48 h or longer, until all the formalin is removed. Test this by placing one of the disks on a nutrient agar plate that has been flood-seeded with a 24 h culture of the Oxford strain of *Staphylococcus aureus*. When virtually no zone of inhibition occurs, the disks are considered free from formaldehyde. Place disks in sterile bottles, cover with sterile distilled water and finally pasteurize by heating at 70°C for 20 min. Store at 4°C. Test a random sample of disks for sterility and stability by incubating in CMB at 37°C for at least 14 days.

Tolerance tests

Growth of the test organism is observed in solid media containing the test agent. We recommend separate plates, one containing sodium taurocholate 0.5% and one with gentian violet.

Prepare the media as follows: 0.25% Yeast extract (Oxoid) and 0.25% sodium succinate are added to the Columbia agar base before autoclaving. When cooled to about 50°C, add haemin 5 μg/ml and menadione 1 μg/ml. Then add the inhibitory agents as follows: *bile* medium, 0.5% sodium taurocholate; *gentian violet* medium, Gurr's gentian violet (BDH) to a final concentration of 1 in 100 000. Aqueous stock solutions of the dye are prepared at 100× the final concentration, and the sodium taurocholate at 10× the final concentration, and sterilized by autoclaving at 121°C for 20 min. The control medium has no further additives.

Each medium is seeded with 0.02 ml of starter CMB culture, and this inoculum is spread with a loop over about 1/6 of the area of the plate. After incubation, plates are observed for growth or inhibition. Set up known control organisms on each batch of tolerance plates. Suitable control strains, and the expected results are as follows:

Test organism	Growth on medium		
	Control	with sodium taurocholate	with gentian violet
B. fragilis (NCTC9344)	+	+	–
B. intermedius (NCTC9338)	+	–	–
F. necrogenes (NCTC10723)	+	+	+

Antibiotic resistance tests

These tests are useful in the characterization of some anaerobes. Note that some of the substances are used at levels that could not be safely achieved therapeutically; these tests should not be confused with antibiotic *sensitivity* tests.

Freshly prepared blood agar plates are seeded by spreading 0.02 ml of starter CMB culture on the surface with a glass spreader. The diameters of zones of inhibition are measured after 24 h incubation, or when satisfactory growth occurs. A zone of <15 mm is considered to indicate resistance.

The following antibiotic disks should be used: neomycin, 1000 μg/disk; kanamycin, 1000 μg/disk; benzylpenicillin, 2 units/disk; metronidazole, 5 μg/disk.

Table 36.1 Tests required to identify anaerobic bacteria.
Key: N, necessary; . . ., not necessary; U, use in some cases. For *Methods* see text, Chs 36 and 37. BA, blood agar plate; EYA, egg-yolk agar plate; CMB, cooked meat broth; PPY, proteose peptone-yeast extract broth; TG, thioglycollate medium; SSA, semi-solid agar.

Test	Test required for identification of				Media employed (*see Key*)
	Bacteroides	*Fusobacterium*	*Clostridium*	Anaerobic cocci	
No growth in air	N	N	N	N	BA
No growth in air + 10% CO_2	N	. . .	. . .	N	BA
Gram stain reaction	N	N	N	N	CMB or PPY or BA
Cell morphology	N	N	N	N	CMB or PPY or BA
Spore shape and position	. . .	. . .	N	. . .	CMB
Motility	. . .	. . .	U	. . .	CMB or SSA or BA
Colonial morphology	N	N	N	N	BA
Colony fluorescence at 365 nm	U	. . .	U	. . .	BA
Pigment	N	. . .	. . .	U	Lysed BA or BA
Phospholipase, and neutralization by antisera	. . .	. . .	N	. . .	EYA
Lipase	. . .	U	N	. . .	EYA
Bile and dye tolerance	Sodium taurocholate Gentian violet		. . .	. . .	Tolerance test agar
Antibiotic resistance	Kanamycin 1000 μg Neomycin 1000 μg Penicillin 2 units Metronidazole 5 μg		. . .	Metronidazole 5 μg Novobiocin 5 μg Vanomycin 5μg Liquoid 100 μg	BA
Indole production	N	N	N	N	PPY
Aesculin hydrolysis	N	N	N	U	CMB + 1% aesculin or PPY + 0.2% aesculin
Gelatin digestion	N	. . .	N	. . .	CMB with charcoal gelatin disk
Meat digestion	. . .	. . .	U	. . .	
Fermentation[a]	Glucose Lactose Sucrose Maltose Rhamnose Trehalose Xylose Xylan	Glucose Lactose Sucrose Mannose Starch Fructose	Glucose Lactose Sucrose Maltose Mannose Mannitol Xylose Melibiose Inositol	Glucose Lactose Sucrose Maltose	PPY or TG
Urease	N	. . .	U	. . .	PPY or TG
Nitrate reduction	U	. . .	. . .	U	PPY with nitrate
Toxin test	. . .	. . .	U	. . .	
Gas chromatography	U	U	U	U	PPY + 1% glucose

[a] The extended range of these useful sugars is not always necessary. The selection depends upon the degree of characterization required (see following tables).

Table 36.2 Typical patterns of results obtained in antibiotic-disk resistance tests and tolerance tests with anaerobic Gram-negative rods.
Key: R, resistant; S, sensitive; S/R, 30–70% of strains give each result; R(S), few species give sensitive result; +, growth; −, inhibited; −(+), few species give positive result; +/−, result varies depending on species.

Test	Pattern of results obtained with strains of				
	B. fragilis group	*B. melaninogenicus/oralis* group	Asaccharolytic *Bacteroides* group	*Fuso-bacterium*	*Leptotrichia buccalis*
Antibiotic resistance[a]					
Neomycin 1000 μg	R	S	S/R	S	S
Kanamycin 1000 μg	R	R	R(S)	S	S
Penicillin 2 units	R	S/R	S	S	S
Tolerance of:					
Bile salt[b]	+	−	−	+/−	−
Gentian violet[b]	−	−	−(+)	+	+
Fermentation of:					
Glucose	+	+	−	+/−	+

[a] Amount of antibiotic in disk is given.
[b] See *Methods*.

Table 36.3 Identification of anaerobic bacteria to genus level by gas chromatography, cell morphology and Gram-stain reaction.

Gram negative		**Gram positive**	
Rod-shaped bacilli		*Rod-shaped bacilli*	
a. Peritrichous flagella or non-motile		a. No spores present	
1. Lactic acid only major product	*Leptotrichia*	1. Lactic acid only major product	*Lactobacillus*
2. n-Butyric acid with no (or only trace amounts of) iso-butyric or iso-valeric acid	*Fusobacterium*	2. Lactic and acetic acids in ratio 1: > 1	*Bifidobacterium*
3. Not as (1) or (2) above, and usually acetic and succinic acid produced	*Bacteroides, Capnocytophaga*[a]	3. Acetic and propionic acids as major products	*Propionibacterium*
b. Polar flagella, motile	*Campylobacter*[a]	4. Acetic acid ± formic acid, and lactic or succinic acid or both as major products	*Actinomyces*
c. Spiral cells with axial fibrils	*Treponema, Borrelia*	5. n-Butyric acid + others, or acetic and formic acid, or no major acids produced	*Eubacterium*
Cocci		b. Spores present	*Clostridium*
1. Acetic and propionic acid	*Veillonella*	*Cocci*	
2. Acetic and butyric acid	*Acidaminococcus*	1. Lactic acid only major product	*Streptococcus*[a]
3. Iso-butyric, butyric, iso-valeric and caproic acid	*Megasphera*	2. Not as (1)	*Peptococcus, Peptostreptococcus, Ruminococcus*

[a] Although not strict anaerobes, these organisms are included because on primary isolation they may fail to grow aerobically or may grow only anaerobically. They are resistant to metronidazole.

Table 36.4 Differential characters and key for the identification of the *Bacteroides fragilis* group.
Key: +, 95% of strains give a positive result; −, 95% of strains give a negative result; +/−, 30–70% of strains give each result; +(−), 70–95% of strains give the positive result; . . ., not tested. For *Methods* see text. Key reactions are printed in bold.

Bile tolerance	Aesculin hydrolysis	Glucose[a]	Lactose[a]	Indole production	Sucrose[a]	Trehalose[a]	Rhamnose[a]	Xylan[a]	Fatty acids[b] produced	Species
+	+	+	+	+	+	+	+	+	A S (p ib iv l)	*B. ovatus*
+	+	+	+	+	+	+	+	−	A S (f p ib iv l)	*B. thetaiotaomicron*
+(−)	+	+	+	+	+	+(−)	−	. . .	A p S (f ib iv l)	*B. uniformis*
+/−	+	+	+	+	+	−	+	+/−	A p S (iv l)	*B. variabilis*
+(−)	+	+	+	+	+	+(−)	−	. . .	A p S (f ib iv l)	*B. uniformis*
+	+	+	+	+	−	−	+	+	A p S (ib iv l)	*B. eggerthi*
+	+	+	+	+	−	−	−	. . .	A p ib b iv S (l)	*B. splanchnicus*
+	+	+	+	−	+	+	+(−)	−	A S (p ib iv l)	*B. distasonis*
+	+/−	+	+	−	+	−	+	−(+)	A p S (ib iv l)	*B. vulgatus*
+	+	+	+	−	+	−	−	−	A p S (f ib iv l)	*B. fragilis*

[a] Fermentation test.

[b] Fatty acids in gas chromatography. Symbols outside brackets, produced by all strains; symbols inside brackets, produced by some strains. The following symbols are used to identify the acids and to indicate the relative amounts produced:

Acid	0.2–10 μmol/ml	>10 μmol/ml
Acetic	a	A
Formic	f	F
Propionic	p	P
Iso-butyric	ib	iB
Butyric	b	B
Iso-valeric	iv	iV
Valeric	v	V
Iso-caproic	ic	iC
Caproic	c	C
Heptanoic	h	H
Lactic	l	L
Succinic	s	S
Phenylacetic	ph	Ph

Table 36.5 Differential characters and key for the identification of the *Bacteriodes melaninogenicus/oralis* group.
Key: See Table 36.4.

Glucose[a]	Maltose[a]	Xylose[a]	Indole production	Sucrose[a]	Pigment production	Aesculin hydrolysis	Lactose[a]	Fatty acids produced	Species
+	+	+	+	+	−	+	+	A p iv S	*B. zoogleoformans*
+	+(−)	+	−	+	−	+/−	+	A S (p ib iv l)	*B. ruminicola*-like[b]
+	+(−)	−	+	+(−)	+	−	−(+)	A iv S (p ib l)	*B. intermedius*
+	+	−	−	+	+	+/−	+	A S (p ib iv l)	*B. melaninogenicus*-like[c]
+	+	−	−	+	−	+/−	+	A S (p ib iv l)	*B. oralis*-like[d]
+	+	−	−	−	+	−	−	A ib iv S (l)	*B. corporis*
−(+)	−	−	−	−	−	+	−	a s (pl)	*B. capillosus*[e]
+	+	−	−	−	−	−	+	A iv S (p ib l)	*B. bivius*
+	+	−	−	−	−	−	−	A S (p ib iv l)	*B. disiens*

[a] Fermentation test.
[b] Includes *B. ruminicola, B. buccae* and *B. oris*.
[c] Includes *B. melaninogenicus, B. loeschi* and *B. denticola*.
[d] Includes *B. oralis, B. veroralis* and *B. buccalis*.
[e] Asaccharolytic strains occur; see Table 36.6.

Table 36.6 Differential characters and key for the identification of the asaccharolytic *Bacteroides* group.
Key: see Table 36.4.

Glucose[a]	Indole production	Nitrate reduction	Motility	Aesculin hydrolysis	Urease production	Pigment production	Phenylacetic acid production	Fatty acids produced	Species
−(+)	−	−	−	+	−	−	−	a s (p l)	*B. capillosus*
−	−	+	−	−	+	−	−	a s (f p l)	*B. ureolyticus*
−	+(−)	−	−	−	−	+	+	A p ib B iv ph (l s)	*B. gingivalis*
−	+(−)	−	−	−	−	+	−	A p ib B iv (l s)	*B. asaccharolyticus*-like[b]
−	−	+	+	−	−	−	−	A p iB B iV (f l s)	*B. praeacutus*

[a] Fermentation test.
[b] Includes *B. asaccharolyticus* and *B. endontalis*

Table 36.7 Differential characters and key for the identification of the fusobacteria and *Leptotrichia buccalis*.
Key: see Table 36.4.

Indole production	Aesculin hydrolysis	Mannose[a]	Starch[a]	Sucrose[a]	Fructose[a]	Lipase production	Glucose[a]	Lactose[a]	Butyric acid[b]	Fatty acids produced	Species
+	–	–	–	–	+(–)	–	–	–	+	a p B s (f L)	*F. nucleatum*
+	–	–	–	–	–(+)	+(–)	–(+)	–	+	a p B (f L s)	*F. necrophorum*
+	–	–	–	–	–	–	+(–)	–	+	a p B (f L s)	*F. gonidiaformans*
+	–	–	–	–	–	–	–(+)	–	+	a p B L (f s)	*F. naviforme*
+(–)	–	+	–	–	+	–	+	–	+	A B L (p s)	*F. varium*
–	+	+	–(+)	+/–	+	–	+	+	+	a p B (f iv L s)	*F. mortiferum*
–	+	+	–(+)	+	+	–	+	+	–	L (a f s)	*L. buccalis*
–	+	+	–	–(+)	+	–	+	–	+	a B (f p l s)	*F. necrogenes*
–	+	–	–(+)	–(+)	+(–)	–	+(–)	+(–)	+	B (a f p L s)	*F. prausnitzi*
–	–	–(+)	+	–	–(+)	–	+	–	+	a B L s	*F. plauti*
–	–	+	–	+	+	–	+	–	+	a B L	*F. perfoetans*
+(–)	–	+	–	–	+	–	+	–	+	A B L (p s)	*F. varium*
–	–	–	–	–	–	–	–	–	+	a B (f L s)	*F. russi*

[a] Fermentation test.
[b] Butyric acid is a major product of the fusobacteria.

Table 36.8 Differential characters and key for the identification of the anaerobic cocci. See *Methods* at end of chapter.
Key: S, sensitive; R, resistant; S(R), a few strains give resistant result; See also Table 36.4.

Sensitivity or Resistance to					Fermentation of				Production of			
Metronidazole	Vancomycin	Novobiocin	Liquoid	Indole production	Glucose	Lactose	Sucrose	Maltose	Caproic acid	Butyric acid	Fatty acids	Species
S	S	S	R	−	+	+	+	+	−	−	A s (l p)	*Peptostreptococcus productus*
S	S	S	R	−	+	+	−	+	−	−	a L (s)	*Peptostreptococcus parvulus*
S	S	S	S(R)	−	+	−	−	+	−	+/−	A (ib b iv ic l)	*Peptostreptococcus anaerobius*
S	S	S	R	−	−	−	−	−	−	−	A (f l s)	*Peptostreptococcus micros*
S	S	S	R	+	−	−	−	−	−	+	a p b	*Peptostreptococcus indolicus*
S	S	R	R	+	−	−	−	−	−	+	a b	*Peptostreptococcus asaccharolyticus*
S	S	R	R	−	+	−	−	−	−	−	A F	*Peptostreptococcus saccharolyticus*
S	S	R	R	−	−	−	−	−	+	+	ib B iv C (a l s)	*Peptococcus niger*
S	S	R	R	−	−	−	−	−	−	+	A (f l s)	*Peptostreptococcus prevotii*
S	S	R	R	−	−	−	−	−	−	−	a b (f p l s)	*Peptostreptococcus magnus*
S	R	...	...	−	−	−	−	−	−	−	a p (s)	*Veillonella parvula*[a]

[a] *V. parvula* and related Gram-negative species (see Mays et al 1982).

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Clostridia of wound infection

The genus *Clostridium* is composed of anaerobic large, straight or slightly curved Gram-positive spore-bearing rods, 3–8 × 0.6–1 μm with slightly rounded ends. Gram-variable, Gram-negative and pleomorphic forms are common. A few species can grow poorly aerobically.

General recommendations for anaerobic culture procedures are given in Chapter 7, and principles for the identification of anaerobes are outlined in the first part of Chapter 36, before the systematic treatments of Gram-negative anaerobes and anaerobic cocci. Details of special relevance to the clostridia are given in this chapter and in Chapter 38. For convenience, we deal here with the clostridia that may be involved in wound infections, with brief accounts of the histotoxic group; *Clostridium tetani* and the laboratory diagnosis of tetanus are dealt with separately at the end of this chapter. Enteropathogenic clostridia are discussed in the following chapter.

Identification of clostridia

In practice the routine characterization of pathogenic clostridia is often limited to a few tests that will presumptively indicate the likely species; this simple approach partly explains why many laboratories have experience of a relatively narrow range of clostridial species. Accurate subdivision of the clostridia to species level rests upon morphological criteria, the results of biochemical tests and gas chromatography (GC), together with the identification of some specific toxins (see, e.g. Table 37.1, and see Willis, 1969; Willis, 1977; and Willis & Phillips, 1983). Our approach parallels that adopted in Chapter 36 and is based on a series of simple tests that allow precise characterization. Initial steps involved in characterization of clostridia and the range of tests of value in detailed identification are given in Chapter 36 (see Tables 36.1 and 36.3). Thereafter, the clostridia can be divided into four groups based on lecithinase production, and lactose and glucose fermentation (see Tables 37.2–37.5, at end of chapter). Whenever possible, the identity of toxigenic species should be confirmed by specific toxin-neutralization tests, although it should be borne in mind that non-toxigenic strains may also occur. Even without GC or animal tests, presumptive identification is often possible. (*Note*: as in Ch. 36, key reactions in the tables that give differential characteristics of important pathogenic species are indicated in bold and these provide a short-cut identification system for the clostridia.)

Many of the techniques used for identification of clostridia are also used for other anaerobes and detailed instructions are given in Chapter 36; tests and procedures of particular relevance to clostridia are included here.

All clostridia produce spores but they vary markedly in their readiness to do so: prolonged incubation of cooked meat broth (CMB) cultures may be required. The spores are most readily demonstrated in wet films examined by phase contrast microscopy. The shape of the spore and its position in the bacillus is of some help in classification. *C. perfringens* and the type species *C. butyricum* produce capsules. Most clostridia are motile, but *C. perfringens* is not. Some motile species do not show active motility under the relatively aerobic conditions of the usual wet film preparations and it may be advantageous to use a semi-solid agar stab (see *Methods*

section below). The active motility of species such as *C. septicum* and *C. sporogenes* may be of advantage or of distinct disadvantage in the isolation of pure cultures on solid media (see below).

Biochemical tests are important in the classification of many clostridia. *C. perfringens*, *C. septicum*, *C. tertium* and *C. fallax* are predominantly saccharolytic; *C. sporogenes* and *C. histolyticum* are actively proteolytic and *C. tetani* is slightly proteolytic. These activities are reflected in the cultural appearances in CMB (and also in litmus milk medium). The *saccharolytic* clostridia grow rapidly and vigorously in carbohydrate media with production of acid and abundant gas; detailed recommendations for specific sugar fermentation tests are given in the *Methods* section of Chapter 36 (and see Table 36.1). When grown in CMB, saccharolytic clostridia rapidly produce acid and gas but they do not digest the meat; the cultures may have a slightly sour smell and the meat is often reddened. Gas production is not necessarily indicative of sugar fermentation, as proteolysis may be accompanied by evolution of gas bubbles.

The *proteolytic* clostridia digest protein and liquefy gelatin and coagulated serum. Cultures in meat medium produce blackening of the meat, decomposing it and reducing it in volume with the formation of foul-smelling products.

In *litmus milk medium* (Ch. 8), *C. perfringens* produces acid and gas. The acid clots the milk and the gas breaks up the clot, resulting in the 'stormy clot' reaction that is produced by almost all strains of *C. perfringens* but is not specific for this organism as various mixed cultures may mimic the reaction. Litmus milk medium does not support luxurious growth of some clostridia. It is of very limited usefulness in a diagnostic laboratory.

Phospholipase-C (lecithinase) and *lipase* activity can be demonstrated when cultures are grown on egg-yolk media (see *Methods* section below). Zones of opacity produced in egg-yolk agar cultures of phospholipase-producing clostridia can be neutralized by antisera prepared against *C. perfringens* type A and *C. novyi* type A toxins (the Nagler effect). The specificity of this neutralization is valuable in identification of a range of clostridia (see Table 37.2 at the end of this chapter).

Gas chromatography (GC) is also of value in the identification of clostridia. GC used in conjunction with the Gram stain reaction and cell morphology can identify anaerobes to generic level (see Table 36.3). The production of butyric acid by many species of clostridia readily differentiates them from some other genera of anaerobic Gram-positive rods (*Lactobacillus*, *Bifidobacterium*, *Actinomyces* and *Propionibacterium*). However, it is necessary to demonstrate spores to differentiate the non-butyric-acid-producing clostridia from these genera, and the butyric-acid-producing clostridia from *Eubacterium* species.

GC, used in conjunction with a suitable range of phenotypic tests, can also help to identify some clostridia to species level. This is especially so when the phenotypic characteristics are similar for more than one species, or when differentiation of the species depends on the results of tests that require prolonged incubation (gelatin liquefaction, spore production), or on tests that give variable results if not done under optimal conditions (motility). Thus, butyric acid production differentiates lecithinase positive strains of *C. subterminale* from *C. limosum*, and *C. butyricum* from *C. clostridiforme; C. irregularis*, *C. cochlearium* and lecithinase negative strains of *C. subterminale* can be differentiated by butyric and isobutyric acid production; isobutyric acid production differentiates *C. difficile* from *C. innocuum* and *C. putrificum* from *C. novyi* type C (see Tables 37.2–37.5, at the end of this chapter). Recommendations for GC technique are given in the *Methods* section of Chapter 36.

CLOSTRIDIUM PERFRINGENS

C. perfringens (*C. welchi*) can be identified as outlined in Table 37.2 (see end of this chapter). Five types (A–E) are distinguished by the different combinations of major lethal toxins that they produce (Table 37.1). *C. perfringens* type A occurs normally in numbers of about 10^4/g wet

Table 37.1 The major lethal toxins and minor lethal or non-lethal factors produced by the various types of *Clostridium perfringens* (after Brooks et al 1957).
Key: +, produced by >95% of strains; +, produced by most strains; (+), produced by some strains; −, not produced.

Type	Occurrence	Major lethal toxins				Minor lethal or non-lethal factors							
		α	β	ε	ι	γ	δ	η	θ	κ	λ	μ	ν
A1	Gas gangrene Puerperal infection Septicaemia	+	−	−	−	−	−	(+)	(+)	(+)	−	(+)	(+)
A2	Food poisoning	+	−	−	−	−	−	−	(+)	(+)	−	(+)	+
B	Lamb dysentery	+	+	+	−	+	(+)	−	+	−	+	+	+
C	'Struck' in sheep	+	+	−	−	+	+	−	+	+	−	−	(+)
	Enteritis in other animals	+	+	−	−	?	−	−	+	+	−	−	(+)
	Enteritis necroticans in man	+	+	−	−	+	−	−	−	−	−	−	+
D	Enterotoxaemia of sheep and pulpy kidney disease	+	−	+	−	−	−	−	+	+	+	(+)	(+)
E	Doubtful pathogen of sheep and cattle	+	−	−	+	−	−	−	+	+	+	(+)	(+)

weight of faeces in the large intestine of healthy man and animals; this is a median figure and the range is very wide (see Collee 1974). The organism also occurs commonly in soil, water and dust and is particularly associated with manured cultivated soil. The classical *C. perfringens* of gas gangrene belongs to type A1. Note that *C. perfringens* food poisoning is dealt with separately in Chapter 38.

Morphology and staining

A relatively large Gram-positive rod, about 4–6 × 1 μm, with stubby ends, occurring singly or in pairs; often capsulate in tissues. In sugar media the rods are shorter; in protein media they may become filamentous. Non-motile. Spores formed, usually in small numbers and not in the presence of fermentable carbohydrates; typically oval, subterminal or central and not bulging. Spores are produced in special media such as Ellner medium,* but here many bizarre forms occur and sporulation is variable, even with different cultures of the same strain. The improved sporulation media described by Duncan & Strong (1968)* and Phillips (1986)* are now recommended.

* Refer to *Methods* at end of this chapter.

Cultural characters

Anaerobic, but may grow microaerophilically. Optimum temperature range 37–45°C. Grows best on carbohydrate-containing media such as glucose blood agar. Surface colonies large, smooth, regular, convex, slightly opaque disks. On horse blood agar, colonies usually surrounded by a variable zone of complete haemolysis and a wider darker zone of incomplete haemolysis. Other types of colonies include one with a raised opaque centre and a flat radially striate transparent border. Rough flat colonies with an irregular edge resembling a vine leaf may also occur. A variant occasionally produces very mucoid broth cultures and tenacious colonies on blood agar.

Biochemical reactions

Actively saccharolytic. Ferments, with gas production, glucose, lactose, sucrose, maltose, mannose, inositol, starch and (some strains) salicin, glycerol and inulin; mannitol and galactitol (dulcitol) not fermented (see Ch. 36 for *Methods*). Acid and gas produced in litmus milk medium gives the 'stormy clot' reaction; this is produced by almost all strains but is not specific for this organism. The culture has a sour, butyric

acid odour. Gelatin liquefied; coagulated serum not usually liquefied. In CMB the meat is reddened but not digested. Hydrogen sulphide is produced; sulphite is actively reduced; most strains reduce nitrates to nitrites.

Viability

Spores generally resist routinely used antiseptics and disinfectants with the exception of formaldehyde and glutaraldehyde. The spores of classical type A1 strains do not survive boiling for more than a few minutes, whereas the spores of type A2 food-poisoning strains and certain type C strains may be markedly heat resistant (see Ch. 38).

Vegetative cells of *C. perfringens* are very sensitive to heat and disinfectants. *C. perfringens* is sensitive to penicillin, erythromycin, many cephalosporins and metronidazole. Generally sensitive to clindamycin. Typically resistant to aminoglycosides.

Toxins

The five types of *C. perfringens* can be differentiated by their production of the four major lethal toxins (Table 37.2). Typing is done by a combination of in-vitro and in-vivo neutralization tests; for details see Volume 2 of the 12th Edition of this book (p 474).

Alpha (α) toxin is produced by all types but notably by type A1 strains; occasional negative strains occur. It is lethal for laboratory animals and necrotizing on intradermal inoculation. The α toxin is a Ca^{2+} or Mg^{2+}-dependent phospholipase, or lecithinase-C (E.C. 3.1.4.3; phosphatidylcholine choline phosphohydrolase). In the presence of free Ca^{2+} or Mg^{2+} it produces opalescence in serum or egg-yolk preparations by splitting phospholipid complexes. The reaction can be inhibited by specific antitoxin; this is the basis of the Nagler test.*

The enzyme is haemolytic for the red cells of most species except the horse and the goat. The clear zones of haemolysis typically seen around colonies of classical type A1 strains grown on horse blood agar are produced by the theta (θ) toxin and not by the α-toxin. With the red cells of the sheep in particular, the α toxin provides an example of a 'hot-cold' lysin. Activity may be assayed by turbidity tests with egg-yolk emulsion (lecitho-vitellin, LV) or human serum as indicator, or by haemolysis tests incorporating antisera to other haemolytic toxins that may be produced by *C. perfringens*.

Animal pathogenicity

Virulence for guinea-pigs can be demonstrated by subcutaneous or intramuscular injection of 0.5–1 ml of a 24 h culture in CMB into the thigh. A control animal can be protected by an injection of 300–500 units of *C. perfringens* antitoxin given intraperitoneally 24 h before challenge. In the test animal, a spreading inflammatory oedema develops with gelatinous exudate and gas production in the tissue planes; the affected muscles become pink, sodden and necrotic and virtually liquefy at the site of injection. The products of growth in a young culture increase the organism's aggressiveness; washed organisms are practically non-pathogenic. An equal volume of a sterile 5% solution of calcium chloride mixed with the inoculum just before injection increases the pathogenicity of a strain.

CLOSTRIDIUM NOVYI

Four types of *C. novyi* (*C. oedematiens*) types A, B, C and D are specifically differentiated on the basis of the toxins or 'soluble antigens' that they produce; they can be identified as outlined in Tables 37.2 and 37.4 (at the end of this chapter). Type A strains and occasionally type B strains may be associated with a severe form of gas gangrene in man.

Morphology and staining

Large Gram-positive rods (5–8 × 0.8–1 μm) with rounded ends and peritrichous flagella. Spores oval, central or subterminal.

Cultural characters

Type A strains are very strictly anaerobic. Type

B and type C strains are even more exacting, and type D strains are among the most exacting of the strict anaerobes that can be cultured as a routine on solid media. All types may be grown satisfactorily in freshly made CMB. Special solid media are required for reliable surface growth of types B, C and D (see Collee et al 1971); these types are not typically associated with human disease. The following observations relate to type A strains, which will grow on appropriate solid media if the atmosphere is strictly anaerobic; growth is enhanced by 10% CO_2. Surface colonies are raised, opaque, sometimes dome-shaped and circular in very young cultures, but often flattened, large and irregular in older cultures. The colonies tend to fuse and form a spreading, sometimes swarming, growth; tracks produced by motile daughter colonies may be seen. Discrete colonies show two zones of haemolysis on horse blood agar – a narrow inner zone of complete haemolysis, and an outer zone of partial lysis; this is not seen with spreading growth. On egg-yolk media, the organism's gamma (γ) toxin produces a zone of opacity caused by its phospholipase (lecithinase) activity which can be inhibited by specific antitoxin. In addition, the epsilon (ϵ) toxin (a lipase) produces a pearly layer effect that is more restricted and overlies the colonies.*

Biochemical reactions

C. novyi type A is saccharolytic and mildly proteolytic. Ferments glucose with gas production. Some strains ferment maltose and inositol. Various other sugars including lactose, sucrose, fructose and mannose are not fermented. Changes produced in litmus milk medium are slight and indefinite. Gelatin is liquefied, but milk agar, coagulated serum and cooked meat are not digested. Hydrogen sulphide is produced. Nitrates and nitrites reduced. Type A strains do not produce indole or reduce sulphite (Rutter 1970).

Serology

All types seem to share at least one common surface antigen and this is exploited in a direct immunofluorescence procedure for the prompt identification of *C. novyi.**

Animal pathogenicity

An intramuscular injection of 0.2–1 ml of an actively growing culture of *C. novyi* type A in CMB into the thigh muscles of a guinea-pig may cause overwhelming gas gangrene, with minimum gas production but with massive oedema. With some strains there may be difficulty in initiating the infection and the procedure outlined above for *C. perfringens* should be followed.

CLOSTRIDIUM SEPTICUM

C. septicum and *C. chauvoei* are very similar (Table 37.3). Both organisms occur widely in the soil and in animals in health and disease, but *C. septicum* is also well recognized as a pathogen of man. It may be involved in gas gangrene (clostridial myositis) on its own or in association with other clostridia.

Morphology and staining

Moderately large Gram-positive rods with rounded ends, 3–20 × 0.6–1 μm. Pleomorphic; short forms, swollen 'citron bodies' and long curved filaments occur; degenerate Gram-negative forms common. Actively motile with peritrichous flagella. Spores readily formed; oval, central or subterminal and distend the bacillus.

Cultural characters

Obligatory anaerobe but less strict than *C. tetani*. Optimum temperature 37°C. Grows on ordinary media, but glucose promotes growth. Grows well on blood agar to produce irregular transparent colonies, later becoming greyish and opaque with fairly coarse projecting radiations; often confluent spreading growth. Colonies on horse blood agar may show a narrow zone of haemolysis.

Biochemical reactions

Ferments various sugars including glucose,

lactose, maltose and salicin, but not mannitol or sucrose. Slight acid production in litmus milk medium may cause slow clotting. Liquefies gelatin but not coagulated serum; no proteolytic effect on milk agar. The meat in CMB is reddened but not digested. Hydrogen sulphide is produced but not indole. Nitrates reduced to nitrites. Sulphites reduced. *C. septicum* does not produce a phospholipase or lipase effect on egg-yolk agar.

Antigenic characters

Six groups distinguished on the basis of two somatic antigens (1, 2) and five flagellar (H) antigens (a–e). Marked cross-relationship with *C. chauvoei*, which shares a common spore antigen with *C. septicum*. There are good reasons to consider *C. septicum* and *C. chauvoei* as different types within the same species. However, most *C. chauvoei* strains have a distinct specific antigen and this is exploited in a direct immunofluorescence procedure for the prompt identification of these two species by ultraviolet microscopy.*

Soluble antigens

The α toxin of *C. septicum* is lethal, haemolytic and necrotizing. The β toxin is a deoxyribonuclease. The γ toxin is a hyaluronidase, and the δ toxin is an oxygen-labile haemolysin. A fibrinolysin is produced and the organism also produces a haemagglutinin and a neuraminidase.

Animal pathogenicity

Intramuscular injection of cultures into guinea-pigs produces gas gangrene. The organisms invade the blood and the animal dies within a day or two. Smears from the liver show long, filamentous forms and 'citron bodies'.

CLOSTRIDIUM BIFERMENTANS AND CLOSTRIDIUM SORDELLI

C. bifermentans and *C. sordelli* are closely related, but *C. bifermentans* is non-pathogenic whereas pathogenic strains of *C. sordelli* produce a lethal necrotizing toxin and are occasionally associated with wound infections in man. Their identification is outlined in Table 37.2.

Morphology

Short stubby Gram-positive rods with rounded ends, often occurring in chains and showing large oval central spores that do not typically bulge but seem to make the whole organism look bigger. Chains of these forms in Gram smears or in wet films give the appearances of necklaces set with bright beads.

Cultural characters

Grow readily and are relatively non-exacting anaerobes. Growth on blood agar abundant and may spread. Colonies grey-white, convex, roughly circular with irregularly crenated edges; more irregular colonies common. Colonies on blood agar are often, but not invariably, haemolytic.

Biochemical reactions

The name *bifermentans* refers to the ability to decompose both sugars and proteins, and the *sordelli/bifermentans* group shares this double activity with variations: *C. bifermentans* ferments glucose, maltose, mannose, sorbitol and salicin, whereas *C. sordelli* ferments glucose and maltose but not the other sugars in this list; neither ferments lactose nor sucrose. Both are strongly proteolytic; they liquefy gelatin and decompose milk protein, coagulated serum and cooked meat. *C. bifermentans* does not decompose urea, whereas *C. sordelli* usually produces an active urease. Both produce indole and hydrogen sulphide, and both produce a serologically related phospholipase (lecithinase) that is partially neutralized by *C. perfringens* antitoxin.*

Animal pathogenicity

Experimental inoculation of 0.5–1 ml of an actively growing CMB culture of a pathogenic strain of *C. sordelli* into the thigh of a guinea-pig

produces a rapidly lethal oedematous myonecrosis.

CLOSTRIDIUM HISTOLYTICUM

This long slender Gram-positive rod is not a strict anaerobe and can be cultured aerobically. Proteolytic but non-saccharolytic. In meat medium, digestion occurs with the formation of white, crystalline masses of tyrosine. Pathogenic to experimental animals and man. When a culture is injected into the muscle of an animal, in-vivo digestion of the tissues results. Produces a lethal necrotizing exotoxin, an active collagenase, a proteinase, an elastase, an oxygen-labile haemolysin, and various other biologically active products. It may be associated with gas gangrene in man. Its identification is outlined in Table 37.5.

CLOSTRIDIUM TERTIUM

This slender rod is also a non-exacting anaerobe and will grow sub-optimally under aerobic conditions. Weakly motile. Spores oval, terminal. Actively saccharolytic. In litmus milk, acid is formed with gas production and slow clotting. Meat is reddened but not digested. Neither gelatin nor coagulated serum is liquefied. Its pathogenicity is doubtful, but when present in wounds it may give rise to gas production. No exotoxin is produced. See Table 37.3 for an outline of its identification.

CLOSTRIDIUM SPOROGENES

This Gram-positive motile bacillus is very widely distributed. Generally regarded as a harmless saprophyte, but frequently isolated from wound exudates in association with pathogens. Its identification is outlined in Tables 37.2 and 37.4.

Morphology and staining

Usually longer and more slender than *C. perfringens*. Forms abundant oval, central or subterminal spores which may be highly resistant; hence the organism is frequently encountered in mixed cultures in the laboratory, especially after preliminary heating of these cultures to select heat-resistant pathogens. Its spores may survive boiling for periods of 15 min up to 6 h.

Cultural characters

Relatively strict anaerobe. Grows well on simple media, provided that anaerobic conditions are maintained. Surface colonies may present a medusa-head appearance (cf. *Bacillus anthracis*) if the plate is dry. Young colonies small, circular, raised and slightly opaque; soon produce outgrowths and the spreading margin of the colony becomes irregular with coarse feathery projections. On horse blood agar colonies may appear to be haemolytic but this is not caused by a true haemolysin; they are usually irregular and transparent with some central greyish opacity where the colonies are raised. Colonies in shake cultures show as 'woolly' balls of growth. A stab culture develops with lateral spikes like that of *C. tetani*.

Biochemical reactions

Actively proteolytic and saccharolytic; produces amino acids, ammonia, hydrogen sulphide, etc. and cultures have an exceedingly putrid odour. In milk media, casein is precipitated and digested. In CMB, meat is blackened and digested. Gelatin and coagulated serum liquefied. Acid and gas produced from some sugars, including glucose and maltose; lactose and sucrose not fermented.

Laboratory diagnosis of gas gangrene

The bacteriological diagnosis of gas gangrene is usually combined with a general bacteriological examination of the infected wound with which this condition is associated. It is convenient here to give special reference to the recognition of the clostridia, but see also references to bacteroides infections in Chapter 36.

Take exudate from the wound, particularly

from the deeper parts and from parts where the infection seems to be most pronounced. Sterile swabs rubbed over the wound surface and soaked in the exudate are much less satisfactory than pus and excised tissue fragments. If there are sloughs or necrotic tissue or if there is an adequate amount of exudate present in the wound, place good samples of these in sterile screw-capped bottles. If only swabbed specimens are available, ask for at least two or three; one for film preparations, one for aerobic, and one for anaerobic culture. Prompt submission to the laboratory is imperative; desiccation in transit must be avoided. It is good practice to put a swab directly into a screw-capped bottle of CMB at the bedside. The steps in a routine investigation are as follows:

1. Prepare smears for Gram stain. If gas gangrene is present, Gram-positive rods may predominate. Thick, stubby, Gram-positive rods suggest *C. perfringens* or *C. sordelli*; 'citron bodies', boat- or leaf-shaped pleomorphic bacilli with irregular staining, may indicate *C. septicum*; slender rods with round terminal spores suggest *C. tetani; C. novyi* occurs as large rods with oval subterminal spores, but these may be relatively scanty in the wound exudate, even in an active infection. The direct immunofluorescence staining procedure of Batty & Walker (1965) is a most useful diagnostic aid for the prompt identification of *C. septicum* and *C. novyi* and some other clostridia, but not *C. perfringens* (see *Methods* section below).

2. In addition to the media routinely used for detection of aerobes, inoculate a tube of prereduced cooked meat broth (CMB), a blood agar (BA) plate and two plates of selective BA containing neomycin 70–100 μg/ml (see Ch. 36). Primary sensitivity tests can be made at this stage by placing disks, e.g. metronidazole and penicillin, on the first series of streaks.

Consider also the use of: plates containing increased agar (4–6%) to prevent swarming by some species of clostridia;* a selective egg-yolk agar plate, with or without antitoxins, to detect phospholipase- and lipase-producing clostridia, and to demonstrate any Nagler reaction;* additional tubes of CMB, heated at 70°C for 20 min or at 100°C for 5–20 min, to select sporing forms.

3. Incubate the CMB, BA and selective BA anaerobically with 10% CO_2 at 37°C. Leave one selective BA incubating anaerobically undisturbed for 48 h. Examine the other media after 24 h incubation.

4. Examine plates for typical colonies. Comparison of the aerobic and anaerobic plates affords some indication of the presence of strictly anaerobic organisms, but any suspect anaerobe must later be tested in subculture to ensure that it is unable to grow under aerobic conditions (see Watt & Jack 1977). *C. tertium, C. carnis* and *C. histolyticum* can grow to some extent aerobically.

5. Examine CMB enrichment by microscopy, and subculture to plates. In CMB both aerobes and anaerobes flourish; this growth is useful for later subculture should the plate culture fail to yield successful isolation of organisms present in the wound. Film preparations also yield further information on the morphological types of vegetative organisms, and sporing forms that may have developed can be seen.

Note: *C. perfringens* is a common environmental contaminant and, because of its capacity for rapid growth in CMB, it may be present in misleadingly large numbers on secondary plate cultures seeded from primary broth cultures. When it is truly involved in a clostridial myositis (gas gangrene), *C. perfringens* is usually capsulate and can generally be recovered in large numbers on primary plates.

6. Pick suspect colonies and subculture to prereduced CMB or PPY medium for subsequent identification by biochemical tests, and to PPY medium containing glucose 1% for gas chromatography (GC; see *Methods*, Ch. 36).

CLOSTRIDIUM TETANI

C. tetani is a common saprophyte occurring in cultivated soils throughout the world. Tetanus occurs in man and animals when a wound is infected with the organism and conditions permit it to multiply and to produce tetanospasmin, which begins to act on the central nervous

strating the presence of tetanus neurotoxin in culture supernates; a pair of mice is used for each test. Protect one animal by intraperitoneal injection of 500–1500 units (0.5 ml) of tetanus antitoxin 1 h before the test. Inject 0.1 ml of a 48 h CMB culture supernate of the organism intramuscularly into a hind limb of the test and control animals. Signs of ascending tetanus develop in the unprotected animal after several hours; they begin in the inoculated leg and extend to the tail; then the other hind limb is affected and then generalized signs appear. The animal responds to the slightest stimulus with generalized spasms. If large doses of toxin are injected, death may occur in 18–24 h without any of the intermediate symptoms.

Tetanolysin, an oxygen-labile haemolysin, is distinct from the true tetanus toxin.

Laboratory diagnosis of tetanus

The specimen will be wound exudate or tissue removed from the wound.

1. Make a direct smear, Gram stain, and examine for drumstick spore-formers. *Caution*: only a minority of specimens will show these and they are not an invariable index of the presence of *C. tetani*. The use of immunofluorescence staining should be considered here.*

2. Inoculate exudate or homogenized tissue into CMB and on to a BA plate and two selective BA plates (see *Methods* Ch. 36). Incubate anaerobically with 10% CO_2 at 37°C. Consider also seeding an antitoxin-controlled plate for the presumptive identification of *C. tetani*. This involves the use of a freshly prepared BA plate half-smeared with tetanus antitoxin (Lowbury & Lilly 1958). *C. tetani* produces haemolysis which is inhibited by the antiserum. Although there are several objections to this technique, the method is convenient for the provisional screening of large numbers of strains. Confirmation by mouse inoculation is recommended.

3. Leave one selective BA plate undisturbed in the anaerobic atmosphere for 48 h. Check the other plates after 24 h incubation, and daily for up to 4 days. Examine the plates with a hand lens or plate microscope for fine spreading growth. (Note that *C. tetani* type 6 is non-motile). If present, make a pure subculture from the spreading edge to BA and pre-reduced CMB, and incubate anaerobically for 48 h.

4. Examine the CMB enrichment culture daily by microscopy. If clostridial forms are seen, do the following:

a. Heat part of the culture at 80°C for 10 min and then subculture the heated and unheated samples to BA. Incubate the plates anaerobically. (Note that some *C. tetani* spores are heat sensitive.)

b. Direct immunofluorescence staining.

c. Although it is usual to wait until a pure culture is available, toxicity testing can be done with the supernate from the CMB enrichment culture.

5. Check the subculture in CMB. If it is not pure, subculture from the spreading edge of growth on BA to pre-reduced CMB. If the CMB is pure, set up toxin tests in protected and unprotected mice (see above), set up a range of biochemical tests, and examine the fatty acid profile by GC (see Table 37.5).

METHODS

Identification of clostridia by direct staining with fluorochrome labelled antibody

This procedure (see Batty & Walker 1965) is of use for the identification of *C. septicum*, *C. chauvoei*, *C. novyi*, *C. botulinum* types A, B, F, C, D and E, and *C. tetani*. Conjugated immunoglobulins are commercially available from Wellcome.

An air-dried smear is fixed by immersion of the slide in reagent grade anhydrous acetone for 10 min. A drop of the conjugated immunoglobulin is spread on the smear and left at room temperature for 30 min in a humid atmosphere provided by a wet filter paper in a plastic box.

The excess conjugate is quickly rinsed off the smear with phosphate buffered saline at pH 7.6 (NaCl 8.5 g, Na_2HPO_4 1.28 g, $NaH_2PO_4.2H_2O$ 0.156 g, in 1 litre of distilled water); the smear is held in several changes of this buffer during the following 10–15 min. It is then gently blotted dry and mounted with buffered glycerol at pH 8–9 ($NaHCO_3$ 0.0715 g, Na_2CO_3 0.016 g,

water to 10 ml, glycerol to 100 ml) and a glass (not plastic) coverslip for UV microscopy.

Preservation and storage of strains

Strains may be preserved by freeze-drying or by storage in various media. Cooked meat broth containing chalk and minced cooked egg-white is a useful preservation medium (Ch. 6). Germination of spores is sporadic, especially following heat-resistance tests, and this is considered to be partly due to fatty acids in the subculture medium. The inhibitory effect may be reduced by incorporating soluble starch or serum in the medium when subcultures are made from preservation media or from heat-resistance tests.

Media that induce sporulation

Ellner's (1956) medium

This medium is used to induce spore formation in *C. perfringens*. Anaerobiosis may be ensured by heating at 100°C for 10 min and cooling just prior to inoculation. It is important that the inoculum should be adequate; 0.5 ml of an actively growing 4–12 h meat broth culture should be introduced with a pipette into the bottom of the tube of medium. Incubation is in an anaerobic jar at 37°C.

Peptone (e.g. Proteose peptone, Difco)	10 g
Yeast extract	3 g
Soluble starch	3 g
$MgSO_4$	0.1 g
KH_2PO_4	1.5 g
$Na_2HPO_4.12H_2O$	67 g
Water to	1 litre

Steam briefly at 100°C to dissolve, adjust to pH 7.8 with sodium hydroxide 1 mol/litre, dispense in tubes and autoclave at 121°C for 20 min. Tubes should be half to two-thirds full.

Medium of Duncan & Strong (1968)

This medium should be pre-steamed and then cooled to 37°C just before being seeded with a 10% volume of a 4 h active culture of the strain of *C. perfringens* in thioglycollate broth. Addition of activated carbon (1%) may increase the sporulation of some strains. Although high numbers of spores are produced by many test strains, the medium is not invariably successful; this reservation should be applied to all currently developed media for the sporulation of *C. perfringens*.

Proteose peptone	15 g
Yeast extract	4 g
Soluble starch	4 g
Sodium thioglycollate (mercaptoacetate)	1 g
Disodium hydrogen phosphate $Na_2HPO_4.7H_2O$	1 g
Water to	1 litre

The pH of the medium is about 7.0. Steam at 100°C to dissolve, and dispense into screw-capped bottles which should be at least two-thirds full of medium. Autoclave at 121°C for 15 min. After inoculation and incubation, sporulation should be evident within about 6 h; maximum sporulation is likely to be achieved at 10–16 h.

Medium of Phillips (1986)

A plate of this sporulation medium for *Clostridium perfringens* should be seeded by rubbing a dry swab charged with a blood agar culture of the organism over the whole surface. Incubate in an atmosphere of nitrogen 80%, CO_2 10% and hydrogen 10% at 37°C for 48 h.

Blood agar base No. 2 (Oxoid CM27)	39.5 g
Desiccated ox bile (Oxoid L50)	10 g
Sodium bicarbonate, $NaHCO_3$	5 g
Quinoline (BDH No. 30012)	0.5 ml
Defibrinated horse blood	50 ml
Distilled water	1 litre

Dissolve the blood agar base, the ox bile and the quinoline in 800 ml of the water and sterilize at 121°C for 15 min. Cool to 50°C. Add the sodium bicarbonate (dissolved in 200 ml of the water and filter sterilized) at 50°C. Then add the horse blood and pour plates. The pH of the medium should be 8.5.

Alkaline egg medium

This medium promotes spore formation by clostridia. Clostridia remain viable in it for years.

Egg yolk	1
Egg whites	2
Sodium hydroxide, NaOH, 1 mol/litre	6 ml
Water to	500 ml

Beat the yolk and whites, add the sodium hydroxide and water. Heat slowly to 95°C for 90 min, filter through cotton wool and distribute. Sterilize by autoclaving at 121°C for 15 min.

Reinforced clostridial agar

This medium is specially enriched with substances that might promote the growth of clostridia. Its preparation involves the heating of cysteine in the absence of any chemical that could protect it substantially against prompt oxidation, but the medium is nevertheless widely used for the culture of various anaerobes in the following form.

Peptone	10 g
Yeast extract	3 g
Meat extract	10 g
Glucose	5 g
Sodium acetate	3 g
Sodium chloride	5 g
L-Cysteine hydrochloride	0.5 g
Soluble starch	1 g
Distilled water to	1 litre

Steam the ingredients in a flask; filter when dissolved. Adjust pH to 7.4. Add agar 10 g and sterilize by autoclaving at 121°C for 20 min.

Cysteine dithiothreitol blood agar

Moore (1968) described the use of the reducing agents cysteine and dithiothreitol to improve solid media for *C. novyi*, particularly of the more fastidious types B and D. Collee et al (1971) modified Moore's medium by increasing the concentrations of cysteine to 1 mg/ml, dithiothreitol to 0.09 mg/ml and of human blood to 33% and later substituted 10% horse blood for the human blood. This latest modification is described here.

Cysteine-dithiothreitol solution

Cysteine	150 mg
Dithiothreitol	13.5 mg
Distilled water	3 ml

Prepare the solution immediately before use and sterilize by membrane filtration.

Preparation of complete medium

Nutrient agar (Oxoid blood agar base, No. 2)	88 ml
Cysteine-dithiothreitol solution	2 ml
Horse blood	10 ml

Melt the nutrient agar, cool to 45–55°C and add the remaining ingredients, mixing carefully before pouring plates. Dry the plates briefly at 60°C for 10 min to protect the reducing agents. Inoculate and incubate immediately.

Motility

Motility may be difficult to demonstrate with some species and it is advantageous to use more than one method.

1. With some species there is active swarming of the culture on the surface of an anaerobic BA plate.
2. Motility may be demonstrated directly by examining freshly made wet films from log-phase CMB cultures, preferably by phase contrast microscopy under a sealed coverslip.
3. A semi-solid agar stab technique can also be used for demonstration of motility (see Ch. 8). Stab cultures in this medium, freshly prepared with added 1% glucose to enhance anaerobiosis, should be examined frequently before excessive gas production invalidates the test. A non-motile species (e.g. *C. perfringens*) may show lateral spikes of growth along faults extending from the stab line, but the appearance is not likely to be confused with the diffuse growth of a truly motile species.

Control of spreading growth on anaerobic plates

Control of the swarming growth of unwanted organisms in plate cultures presents various problems. Pre-treatment of the proposed

inoculum by differential heating may be effective, but it carries the risk of killing vegetative cells of likely non-sporing and sporing pathogens. Moreover, the spores of some pathogens are not markedly heat resistant and may be inactivated, or their prompt germination may be inhibited. The use of firm agar containing 3–4% agar is recommended as a general control method (see Ch. 8), but colonial morphology is altered. Alternatively, some workers pay particular attention to drying the surface of agar plates to inhibit swarming; but the availability of nutrients for the initiation of colonial growth on a relatively dry surface may be critically impaired. An 'alcohol' plate is sometimes used; here the surface of the unseeded medium is flooded with ethanol, the excess is removed and the plate is exposed in an incubator to dry it at 37°C. The medium is then seeded in the usual manner. If the pathogen to be isolated can grow on MacConkey agar, it should be noted that *Proteus* and *Pseudomonas* species do not spread on this medium. It is sometimes possible to exclude these spreading species by exposing the seeded plate to chloroform vapour for some minutes prior to anaerobic incubation.

Specific serological control of the swarming growth of *C. tetani* and *C. septicum* is possible (see Willis & Williams 1972). When commercial tetanus antitoxin 40–60 units/ml is incorporated into the agar medium, motile strains of *C. tetani* grow as discrete colonies. Similarly, *C. septicum* does not spread on plates on which 0.2–0.5 ml of a *C. septicum* antiserum prepared against the two serological groups is spread.

Tests for phospholipase and lipase; Nagler's reaction

Several clostridia produce phospholipases that give rise to a zone of opalescence extending beyond the colony or line of growth on human serum or egg-yolk media (see Ch. 8). Proteolytic colonies produce zones of clearing on these media, whilst opalescence restricted to the medium underlying a colony and associated with an overlying iridescent 'pearly layer' indicates lipase activity. The phospholipase reaction produced by *C. perfringens* is specifically neutralized by *C. perfringens* antitoxin (Nagler reaction), but the serologically related phospholipases of *C. bifermentans* and *C. sordelli* and some other clostridia are also inhibited. The phospholipase of *C. novyi* type A can similarly be inhibited by specific *C. novyi* antitoxin (see Table 37.2).

For the presumptive detection of *C. perfringens* in direct plate culture, prepare and dry a plate of good quality digest agar containing egg-yolk 5%. On one half of the plate spread 2–3 drops of *C. perfringens* antitoxin (Wellcome) and allow to dry. Then seed with the test organism or with the exudate under investigation, stroking from the antitoxin-free on to the antitoxin-bearing half, and incubate anaerobically at 37°C. On the section containing no antitoxin, *C. perfringens* colonies show a surrounding zone of opacity, i.e. the Nagler reaction, whereas colonies of the organism on the remainder of the plate do not. Parallel stroke cultures of different strains or isolates may be done on one plate, with a known positive control included, for identification of pure cultures.

Lowbury and Lilly's medium

An indicator medium for *C. perfringens* containing Fildes peptic digest of blood to stimulate the production of lecithinase, human serum or egg-yolk to show lecithinase production and *C. perfringens* antitoxin spread over the surface of half the plate to show neutralization of the lecithinase was devised by Hayward (1943) who named it the Nagler plate. Nagler (1945) observed the lipase in addition to the lecithinase reaction in egg-yolk and proposed an egg-yolk medium as indicator of the lecithinase and lipase of *C. novyi*. Lowbury & Lilly (1955) modified the human serum medium by increasing the concentration of agar to prevent the swarming of *Proteus*, making a double layer medium to allow a clearer demonstration of lecithinase reactions and adding neomycin (100 μg/ml) to inhibit lecithinase-producing aerobic sporing bacilli. However in the description given here the concentration of neomycin is only 70 μg/ml, the maximum that is not markedly inhibitory to many clostridia (Collee & Watt 1971). The

neomycin potency of neomycin sulphate is 700 μg/mg. This 70% factor is taken into account in the following recipe.

Agar base

Agar	50 g
Peptone water (Ch. 6)	1 litre

This agar base autoclaved at 121°C for 20 min is used for the lower layer of the medium and as basal medium for the upper layer.

Preparation of complete medium

Agar base	100 ml
Fildes peptic digest of sheep blood	6.5 ml
Human serum, sterile	40 ml
Neomycin sulphate (Upjohn), sterile aqueous solution: 10 000 μg/ml	1.47 ml

The serum may be prepared by treating plasma with 5% of sterile 10% calcium chloride at 37°C. *Note*: Special precautions must be taken when human blood products are used (Chs 7, 15).

Heat the Fildes digest at 55°C for 30 min, melt the agar base and cool it to 56°C. Add the remaining ingredients and pour the medium as the upper layer of double layer plates on a nutrient agar base. Spread 250 international units of *C. perfringens* antitoxin over half of the agar surface.

Willis and Hobbs medium

This medium for the isolation of clostridia (Willis & Hobbs 1959) is a lactose egg-yolk milk agar made selective for various clostridia, particularly *C. perfringens*, by the addition of neomycin. The recommended concentration of 250 μg neomycin sulphate/ml inhibits some clostridia, usually inhibits strains of *Bacillus* and *Staphylococcus*, and greatly reduces the growth of coliform bacilli. As results of culture on Willis & Hobbs medium can be variable and are occasionally confusing, a simpler selective medium such as egg-yolk agar with a good base and neomycin 70 μg/ml is preferred for general use.

Egg yolk suspension. Break eggs with precautions to keep their contents sterile, as described for Löwenstein medium (see Ch. 6), at the same time separating the yolks from the whites. Discard the whites and dilute the yolks with an equal volume of sterile 0.9% sodium chloride solution.

Sterile stock milk. Remove the cream from ordinary milk by centrifuging. Sterilize the skimmed milk by autoclaving at 121°C for 20 min.

Basal medium

Agar	4.8 g
Lactose	4.8 g
Neutral red, 1% solution	1.3 ml
Meat infusion broth, pH 7.0	400 ml
Egg-yolk suspension	15 ml
Milk	60 ml

Dissolve the agar and lactose in the neutral red and broth by steaming, and sterilize at 121°C for 20 min. Cool to 50–55°C and add the egg-yolk and milk. Pour plates.

Possible additions to basal medium

Neomycin sulphate (Upjohn)	250 μg/ml
Sodium thioglycollate	0.1%

Stock sterile solutions of neomycin may be stored in the refrigerator with little loss of potency. The antibiotic is not decomposed by heating at 60°C for 20 min. Thioglycollate may assist the growth of the stricter anaerobes.

Either or both of these reagents may be added at the same time as the egg-yolk and milk.

Table 37.2 Differential characters and key for the identification of the lecithinase-positive clostridia.
Key: +, 95% of strains give a positive result; −, 95% of strains give a negative result; +/−, 30–70% of strains give each result; +(−), 70–95% of strains give a positive result; . . ., not tested. Key reactions are indicated in bold.

Lecithinase production	Inhibition by antitoxin[a]	Lipase production	Indole production	Urease production	Glucose[b]	Lactose[b]	Inositol[b]	Maltose[b]	Motility	Gelatin liquefaction	Iso-butyric acid produced	Fatty acids[c] produced	Species
+	+	+	−	−	+	−	+(−)	+(−)	+	+	−	a P B (f v l s)	*C. novyi* type A
+	+	−	+	+	+	−	−	+	+	+	+/−	A ic (F p ib b iv l s)	*C. sordelli*
+	+	−	+	−	+	−	−	+	+	+	+/−	A F (p ib b iv ic h l s)	*C. bifermentans*
+(−)	+	−	−	−(+)	+	+	+	+	−	+	−	A B L (f p s)	*C. perfringens*
+	+	−	−	−(+)	+	+	−	+	+	+	−	A B (f p L s)	*C. sardiniensis*
+	+	−	−	. . .	+	+	−	+	−	+	−	A B L	*C. absonum*
+	+	−	−	−	+	+	−	+	−	−	−	A B L (f p s)	*C. barati*
−(+)	+	−	−	−	−	−	−	−	+	+	+	A ib b iv (f p l s)	*C. subterminale*
+	+	−	−	−	−	−	−	−	+	+	−	A (f l s)	*C. limosum*
−(+)	−	+	+(−)	−	+	−	+(−)	+(−)	+	+	−	A P B (f v l s)	*C. botulinum* type C
−(+)	−	+	−	−(+)	+	−	−	+	+	+	+	A ib B iv (p v ic s)	*C. sporogenes*
+	−	−	+(−)	−	+	−	+(−)	+	+	+	−	a P B (f v s)	*C. novyi* type B
+	−	−	+	−	+	−	+(−)	−	+	+	−	A P B (l s)	*C. novyi* type D

[a] Observe inhibition of lecithinase by a mixture of *C. perfringens* and *C. novyi* type A antitoxins (see *Methods*).
[b] Fermentation test.
[c] See Table 36.4 for standard key to fatty acid profiles.

Table 37.3 Differential characters and key for the identification of the lecithinase-negative, lactose-positive clostridia.
Key: see Table 37.2.

Lecithinase production	Lactose[a]	Aerotolerance	Indole production	Mannitol[a]	Mannose[a]	Sucrose[a]	Xylose[a]	Spore position[b]	Melibiose[a]	Motility	Butyric acid produced	Fatty acids[c] produced	Species
–	+	+	–	+	+	+	+/–	T	+	+	+	A b L (f s)	*C. tertium*
–	+/–	+	–	–	+	+	–	S	–	+	+	A f B l (s)	*C. carnis*
–	+	–	+	+	+	–(+)	+/–	S/T	–(+)	+	–	A (F l s)	*C. sphenoides*
–	+/–	–	–(+)	–	+	+	+	S	–(+)	+	–	A (F l s)	*C. clostridiforme*
–	+	–	+	–	–	+/–	+(–)	T	–	+	+/–	A F (p b l)	*C. indolis*
–	+	–	–	–	+	+	+	S	+	+	+	A F B (l s)	*C. butyricum*
–	+	–	–	–	+	+	+	S	+/–	+	+	A B (F p l s)	*C. beijerinckii*
–	+/–	–	–(+)	–	+	+	+	S	–(+)	+	–	A (F l s)	*C. clostridiforme*
–	+	–	–	+(–)	+	+	–	T	+	–	–	A F L (p s)	*C. ramosum*
–	+	–	–	–	+	+	–	T	–	+(–)	+	A B L (F s)	*C. paraputrificum*
–	+	–	–	–	+	+	–	S	–	+	+	A F B (l s)	*C. chauvoei*
+(–)	+	–	–	–	+	+	–	S	+/–	–	+	A B L (f p s)	*C. perfringens*
–	+	–	–	–	+	–	–	S/C	–	+	+	A B L (f s)	*C. septicum*

[a] Fermentation test.
[b] T = terminal; S = subterminal; C = central.
[c] See Table 36.4 for key.

Table 37.4 Differential characters and key for the identification of the lecithinase-negative, lactose-negative clostridia.
Key: see Table 37.2.

Lecithinase production	Lactose[a]	Aerotolerance	Lipase production	Aesculin hydrolysis	Mannitol[a]	Sucrose[a]	Xylose[a]	Maltose[a]	Indole production	Toxin test[b]	Spore position[c]	Motility	Iso-butyric acid produced	Fatty acids[d] produced	Species
–	–	–	+	+	–	–	–	+/–	–	+	S	+	+	A p ib B iV (f ic l s)	*C. botulinum* type A and proteolytic types B and F
–(+)	–	–	+	+	–	–	–	+	–	–	S	+	+	A ib B iv (p v ic s)	*C. sporogenes*
–	–	–	+	–	–	+	–	+/–	–	+	S	+	–	A B (f l s)	*C. botulinum* type E and non-proteolytic types B and F
–(+)	–	–	+	–	–	–	–	+(–)	–	+	S	+	–	A P B (f v l s)	*C. botulinum* types C and D
–	–	–	–	+(–)	+	–	–	–	–	+	S/T	+(–)	+	A ib b iv v ic (p c)	*C. difficile*
–	–	–	–	+	+	+(–)	–	–	–	–	T	–	–	A B L (f s)	*C. innocuum*
–	+/–	–	–	+	–	+	+	+	–(+)	–	S	+	–	A (F l s)	*C. clostridiforme*
–	–	–	–	–(+)	–	–	+	+/–	–	–	S	+	+	A ib iV (f p l s)	*C. glycolicum*
–	–	–	–	+	–	–	–	+	–	–	S	+	–	A B (L s)	*C. fallax*
–	–	–	–	–	–	–	–	–	+	–	T	+	–	A B (f p s)	*C. cadaveris*
–	–	–	–	–(+)	–	–	–	–	–	–	T	+	+	A ib B iv (f p ic l s)	*C. putrificum*
–	–	–	–	–	–	–	–	–	–	–	S	–	–	a p B (v)	*C. novyi* type C
–	+/–	+	–	+	–	+	–	+	–	–	S	+	–	A f B l (s)	*C. carnis*

[a] Fermentation test.
[b] Relevant tests indicated in text for different toxins.
[c] S = subterminal; T = terminal.
[d] See Table 36.4 for key.

Table 37.5 Differential characters and key for the identification of the lecithinase negative, asaccharolytic clostridia. *Key*: See Table 37.2.

Lecithinase production	Glucose[a]	Aerotolerance	Spore position[b]	Indole production	Gelatin liquefaction	Toxin test	Meat digestion	Butyric acid produced	Iso-butyric acid produced	Fatty acids[c] produced	Species
−	−	+	S	−	+	−	+	−	−	A (f l s)	*C. histolyticum*
−	−	−	T	+	−	−	−	+	−	A B (f p l s)	*C. malenominatum*
−	−	−	T	+(−)	+	+	−	+	−	A p b (s)	*C. tetani*
−	−	−	S	−	+	+	−	+	+	A ib b iv l	*C. botulinum* type G
−	−	−	S	−	+	−	+(−)	+	+	A p ib B iv (f ic l s)	*C. hastiforme*
−(+)	−	−	S	−	+	−	−	+	+	A ib b iv (f p l s)	*C. subterminale*
−	−	−	S	−	+	−	−	+	−	A p B (f l s)	*C. cochlearium*
−	−	−	S	−	+	−	−	−	+	A ib iv (f p iC l)	*C. irregularis*

[a] Fermentation test.
[b] S = subterminal; T = terminal.
[c] See Table 36.4 for standard key to fatty acid profiles.

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Enteropathogenic clostridia and Clostridium botulinum

This chapter deals with the pathogenic clostridia that affect the bowel. Some of the disease associations are definite and dramatic; others are less clearly defined, and some are still debated.

CLOSTRIDIUM PERFRINGENS FOOD POISONING

Food poisoning caused by C. perfringens type A

Strains of *C. perfringens* type A that produce enterotoxin are associated with a mild form of food poisoning (Vol. 1, 13th Edn, Ch. 36). The so-called 'typical food-poisoning strains' (type A2) that are non-haemolytic or feebly haemolytic on horse blood agar plates and have markedly heat-resistant spores are most frequently encountered, but classical (type A1) strains that are β-haemolytic and have relatively heat-sensitive spores can also be involved (see Table 37.1).

Many opportunities arise for spores of *C. perfringens* to contaminate meat and meat products at the abattoir, in transit to shops and market places, and in catering establishments and in the home. As the organism is encountered so frequently and so widely, it is not usual to attempt to trace the ultimate source in an outbreak of food poisoning but rather to determine the circumstances or conditions that allowed the almost inevitable contamination to be boosted during processing of the food concerned. The heat resistance of type A2 spores allows them to survive the whole cooking procedure, whereas type A1 spores are not likely to cause trouble unless they are introduced by faulty catering practice during the period between cooking and serving.

The bacteriological investigation of *C. perfringens* food poisoning must take account of the findings of Collee et al (1961) and Sutton et al (1971). Virtually 100% of the healthy population carries classical β-haemolytic *C. perfringens* in their gut, and 2–30% may be healthy carriers of heat-resistant *C. perfringens*. The faecal counts of *C. perfringens* just after food poisoning caused by this organism are higher than the counts for healthy people, or for people with diarrhoea due to other causes (median counts 8.5×10^6/g; cf. 1.5×10^4/g in normal subjects). However, it should be noted that the range of counts for commensal *C. perfringens* in normal subjects is wide. The spores of heat-resistant strains often require heat treatment before they grow quantitatively. A semi-quantitative procedure for the isolation of *C. perfringens* from food and faeces is outlined in Figure 38.1. Techniques for identification of *C. perfringens* are discussed in Chapter 37.

Samples of suspect foods should be examined if possible but they are often not available by the time the diagnosis is suggested. The organism will be present as vegetative cells in food and the samples should not be heated before culture. Although the organism does not readily sporulate in routine culture media, it does so in the human gut and considerable numbers of spores of *C. perfringens* may occur in faeces of affected persons. Faecal specimens should be examined for vegetative cells and spores of *C. perfringens*. If faecal samples are boiled before culture, heat-resistant strains will be isolated but the spores of many enterotoxin-producing strains will be killed by this treatment. Mild heating is, however, of value in recovery of heat-sensitive *C. perfringens*

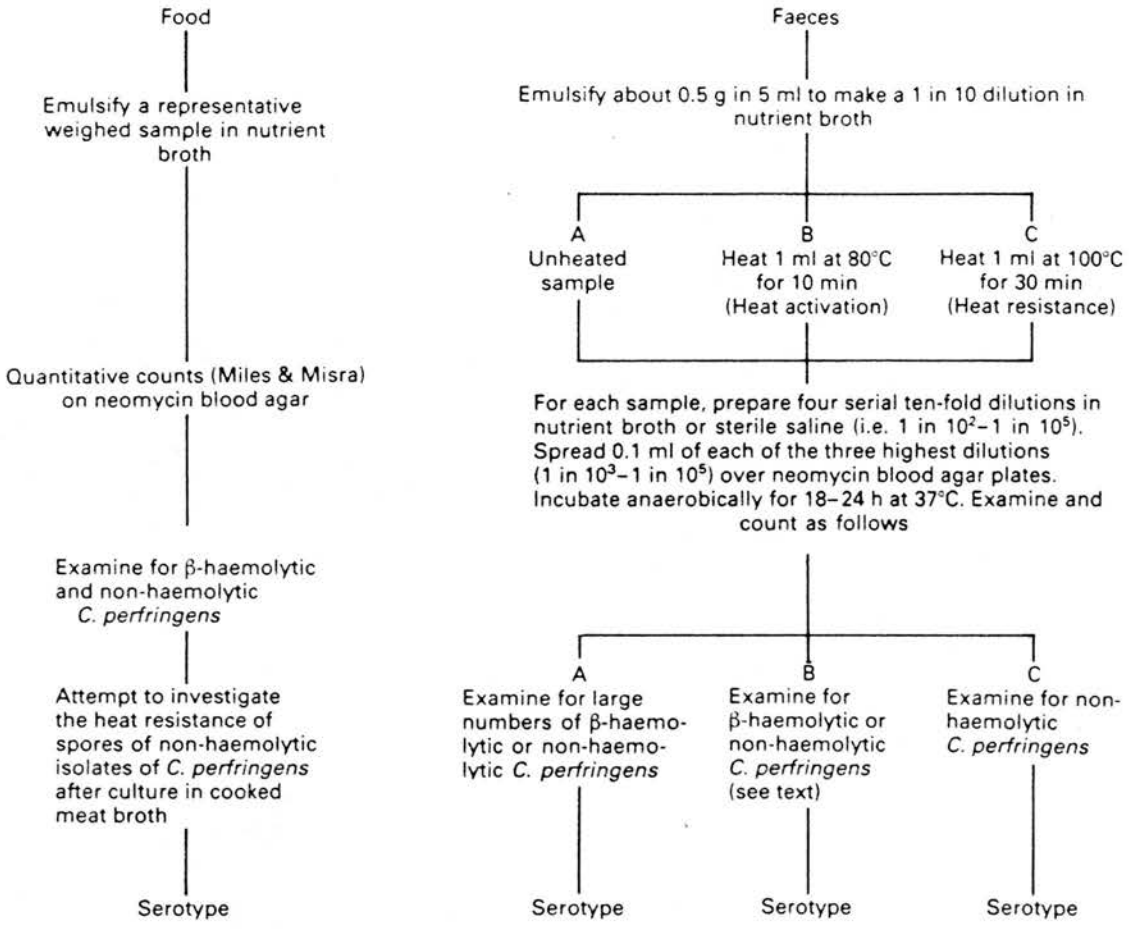

Fig. 38.1 Isolation of *C. perfringens* from food and faeces after a food-poisoning incident.

from faeces as heat shock activates the spores and increases the rate of germination.

The demonstration of *C. perfringens* in the faeces of affected people is of limited value. The qualitative evidence requires to be substantiated by quantitative evidence, because many foods are contaminated with *C. perfringens* and the organism occurs regularly in the faeces of normal people. The count of organisms in remnants of suspect food will depend on the conditions under which the food has been kept and little reliance can be put upon high counts unless the food was held in the refrigerator after serving. Under normal circumstances, counts of the organism do not usually exceed 10^5 per gram of food or faeces, whereas in *C. perfringens* food poisoning the affected food may contain very high counts of *C. perfringens* (up to 10^9 per gram) and the faeces of affected people may contain *C. perfringens* in counts of 10^5–10^8 per gram for some days after the food-poisoning incident.

The approach outlined in Figure 38.1 for examination of faeces includes a mild heat activation step as well as a procedure for selecting the very heat-resistant spores of type A2 strains. The yield obtained from sample A (unheated) represents vegetative cells and some of the spores that may germinate, with either type A1 or A2 strains appearing. Sample B (heat activated) will yield colonies, either type A1 or A2, derived purely from spores, as vegetative cells will not withstand heating at 80°C. Colonies of

C. perfringens grown from sample C (boiled) are derived from markedly heat-resistant spores of type A2 strains.

If a faecal sample yields large numbers of type A1 or A2 colonies, this is compatible with a diagnosis of *C. perfringens* food poisoning, but supportive evidence is really needed. If samples from several people involved in an incident yield the same colony type, the evidence is stronger. If the suspected food yields a similar organism in larger numbers, the case is virtually made.

However, the identity of isolates should be finalized by serotyping, so that a relationship can be established between them. The isolation of classical type A1 β-haemolytic *C. perfringens* from food or faeces must be interpreted with caution if this is not possible. Serotyping of food-poisoning strains by slide agglutination tests is done at the Food Hygiene Laboratory, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT. Food-poisoning strains can be assigned to serotypes 1–75 (Stringer et al 1980) but untypable strains also occur quite frequently.

Send several colonies for each specimen, as strains of more than one serotype of *C. perfringens* may be present in the same specimen. If only one single-colony isolate is examined from each specimen, the identity of serotype may not be observed.

A test has been developed for the direct detection of *C. perfringens* enterotoxin in faeces, food, or culture supernates (Bartholomew et al 1985). A reverse passive latex agglutination kit is now available commercially (Oxoid).

C. perfringens enterotoxin-associated colitis

A syndrome differing substantially from that of *C. perfringens* food poisoning has been reported by Borriello et al (1984). Features include abdominal pain and diarrhoea with blood or mucus in the stools. Vomiting sometimes occurs. Patients are usually elderly; an association with current antibiotic therapy is common but not invariable.

Large numbers of *C. perfringens* (10^7–10^{10}/g) are excreted in the stool, predominantly in spore form, along with the enterotoxin which can often be detected in high titre. The enterotoxin produces a cytopathic effect on Vero cells and can be neutralized by specific antiserum raised against it.

Enteritis necroticans: Clostridium perfringens type C

Enteritis necroticans caused by type C strains of *C. perfringens* (originally designated type F) was recognized in Germany in the late 1940s. The form of the disease associated with pork feasting in the highland natives of Papua New Guinea, called 'pig bel', has been studied in detail (Egerton & Walker 1964). There is segmental involvement of small bowel that may be thickened and tender on palpation; loops of dilated intestine with subsequent fibrinopurulent peritonitis and adhesions and intestinal obstruction develop. The disease is a common cause of death in the native children. *C. perfringens* type C (see Table 37.1) produces heat-resistant spores, which are not so markedly heat-resistant as the original type F German strains, but resist 95°C wet heat for some minutes. The organism produces α-toxin, but it is the β-toxin which causes the disease when produced by organisms multiplying and attaching to the villi of the small gut; the toxin is protected from proteolytic digestion by the simultaneous ingestion of a sweet potato vegetable which contains a protease inhibitor. The low protein diet, and the consequent low intestinal protease activity of the natives, further increase vulnerability to the β-toxin. The organism can be demonstrated in the lesions by special immunofluorescence staining and can be cultured from the intestinal content of patients with the disease. It is also present in the stools of healthy carriers and in environmental samples of soil from native cooking areas where 'pig-bel' is endemic.

CLOSTRIDIUM DIFFICILE

Until recently, this organism was regarded as a component of the normal faecal flora of 40–50%

of neonates, but it was rarely isolated from adults. Since 1977 it has been detected with increasing frequency and is now accepted as a cause of pseudomembranous colitis (PMC) and antibiotic-associated colitis (AAC) (Bartlett 1979). The spectrum of disease caused by *C. difficile* is still not fully known, but it appears to range from PMC through AAC to antibiotic-associated diarrhoea (AAD), and may also include exacerbations of chronic inflammatory bowel disease, post-operative diarrhoea and non-antibiotic-associated diarrhoea (British Medical Journal 1981; Brettle et al 1982). Evidence from animal studies and from the observed clustering of some cases favours the view that infection may be exogenous with colonization of a compromised gut.

Morphology and staining

Rod-shaped (4–8 × 0.5–1 μm) with subterminal or terminal non-bulging oval spores. Gram positive, becoming Gram negative in older cultures. In wet films from cooked meat broth (CMB) cultures it exhibits a characteristic oscillating motility. Electron microscopy shows sparse peritrichous flagella.

Cultural characters

A strict anaerobe. Grows well on blood agar (BA) and cefoxitin cycloserine fructose agar (CCFA) at 37°C. On BA, colonies are glossy, greyish, low convex, roughly circular with an irregular edge sometimes becoming spreading; non-haemolytic. On CCFA medium with blood, colonies are similar to those on BA. On CCFA with egg-yolk and neutral red, colonies at 24 h appear large (2–5 mm), flat to low umbonate, yellow, with a ground-glass appearance and a slightly filamentous edge. In films from this growth, organisms may appear much longer, sometimes almost filamentous, and spores are absent; motility is much reduced. Colonies on CCFA are very susceptible to oxygen and must be subcultured without delay. In solid and liquid media, *C. difficile* produces a characteristic farmyard smell which is unlike that of any other *Clostridium* species.

Biochemical reactions

The biochemical reactions of *C. difficile* are summarized in Table 37.4. Most strains ferment glucose, fructose and mannitol, although we have isolated an asaccharolytic strain of *C. difficile*. Aesculin is hydrolysed and some strains produce gelatinase. All are indole negative and lecithinase negative. Gas chromatography (GC; see *Methods*, Ch. 36) of the volatile fatty acid metabolic products after culture in proteose peptone yeast extract glucose (PPYG) medium gives a characteristic profile (Fig. 38.2) which is diagnostic for an organism that can grow on CCFA medium. *C. difficile* produces *p*-cresol from *p*-hydroxyphenyl acetic acid and this is exploited in the indicator medium developed by Phillips & Rogers (1981) for use with GC.

Toxins

A cytotoxin (toxin B) that causes a cytopathic effect on most tissue cell monolayers is neutralized by certain batches of *C. sordelli* antitoxin. An enterotoxin (Sullivan et al 1982), probably produced by all enteropathogenic strains of *C. difficile*, is only weakly cytotoxic but provokes haemorrhagic dilatation when injected into ligated ileal loops of rabbits; this (toxin A) is thought to be the major virulence factor. In 1987, it was not yet practicable to detect the enterotoxin in the diagnostic laboratory. Detection of the cytotoxin is readily performed; although a few enterotoxin positive, cytotoxin negative strains may be missed, it is a worthwhile addition to culture in investigating a possible *C. difficile* infection (see below).

Sensitivity to chemical and physical agents

Vegetative cells of *C. difficile* are sensitive to oxygen. Spores are resistant to oxygen, to heat (75°C for 10–20 min) and to alcohol, which can be used for their selection. Cresols and phenols have been used as selective agents (e.g. 0.2–0.4% *p*-cresol) but this approach is not recommended (see George et al 1979).

Sensitivity to antimicrobial agents

Most strains are sensitive to low concentrations

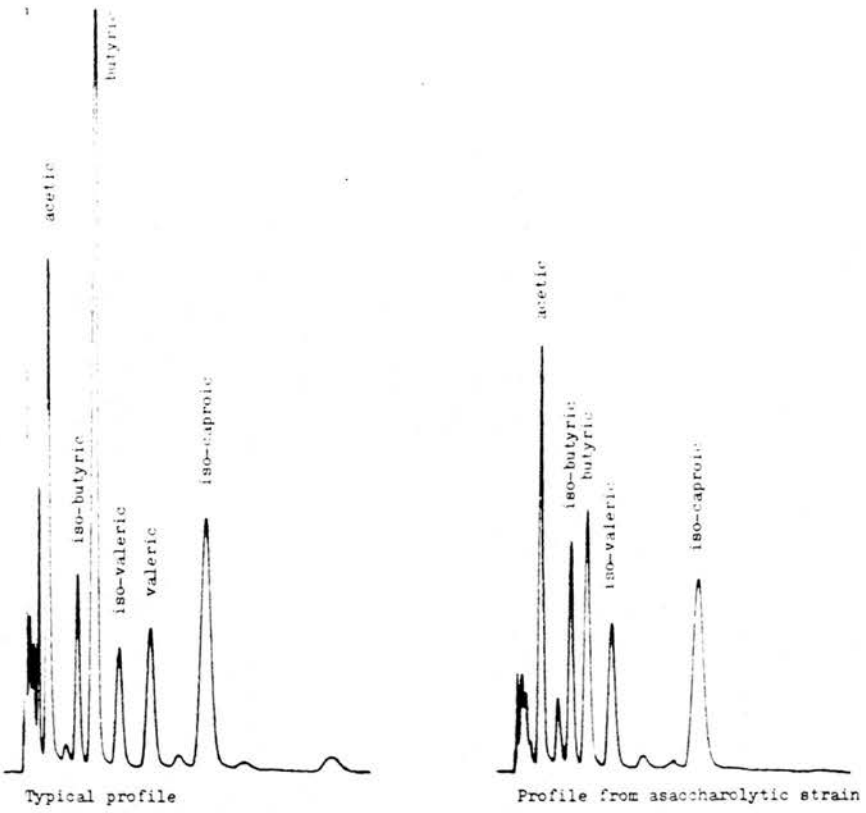

Fig. 38.2 Gas chromatograms of volatile fatty acid metabolic products from 18 h PPYG cultures of a typical and an asaccharolytic strain of *C. difficile* on columns of SP 1220 (see Poxton 1982). *Note*: The small peak between acetic and iso-butyric acids represents propionic acid.

of metronidazole, benzylpenicillin, ampicillin and vancomycin (MIC usually <4 μg/ml, but some up to 32 μg/ml). Most strains are also sensitive to clindamycin, tetracycline and erythromycin, but some are resistant to high levels of these antibiotics. Relatively resistant to aminoglycosides, cephalosporins and cephamycins.

Typing

No generally accepted typing method was available in 1987 when this chapter was written. Various biotypes can be distinguished by a range of simple biochemical tests (Poxton 1982), but this approach is not promising. An immunological fingerprinting method based on visualization of cell surface antigens, after separation on polyacrylamide gel electrophoresis (PAGE), by immunoblot transfer and immunoperoxidase labelling appears more satisfactory (Poxton et al 1984; Sharp & Poxton 1985). A typing method based on PAGE of ^{35}S methionine-labelled proteins has been described by Tabaqchali et al (1986).

Animal pathogenicity

Several investigations of the pathology and epidemiology of *C. difficile* have employed a hamster model. A fatal enterocaecitis can be induced in hamsters if *C. difficile* and antibiotics are given concomitantly. *C. difficile* alone does not usually cause disease but antibiotics alone can result in disease: *C. difficile* is apparently acquired from the environment and then from other animals (Larson et al 1980).

Most strains produce soluble products in broth cultures that are lethal for most laboratory

animals. Intramuscular injection of washed live organisms kills mice. Animal tests are not used for diagnostic purposes at present.

Laboratory diagnosis of C. difficile infection

Send a fresh specimen of faeces or diarrhoeal fluid for examination. The vegetative organisms are sensitive to oxygen and are probably unlikely to survive transportation on a swab. Faeces should be investigated for the presence of cytotoxin*. A direct presumptive diagnostic test (API) based on latex particle agglutination can be applied to a faecal supernate.

The primary assignment is to examine the specimen microscopically and to culture the organism. The introduction of CCFA medium* greatly facilitated the selective isolation of *C. difficile*.

1. Prepare a Gram film and note the presence of any Gram-positive sporing rods. Also prepare a wet film and look for sporing rods showing typical oscillating motility when viewed with phase contrast microscopy. Note the presence of polymorphonuclear leucocytes and red blood cells.

2. Plate out the specimen on fresh CCFA medium, with a large initial inoculum to ensure detection of small numbers of organisms. Incubate the seeded plate anaerobically without delay.

3. Increased yields of *C. difficile* may be obtained by the use of CCFA with reduced concentrations of antibiotics (cefoxitin 8 μg/ml, cycloserine 250 μg/ml) if the sample is previously treated with alcohol to reduce the numbers of non-sporing vegetative commensal species in the inoculum (Borriello & Honour 1981; Levett 1985). The original method used absolute ethanol and homogenized samples were allowed to stand at room temperature for 1 h before plating to selective medium. In our experience, satisfactory results are obtained when a pea-sized sample is homogenized in *c.* 1 ml of industrial methylated spirit; the sample may be plated out within 1–2 min; this makes the method suitable for routine use in the diagnostic laboratory. This procedure gives a significantly higher isolation rate than direct culture on full-strength CCFA.

4. *Enrichment culture*. In addition to primary selective culture, an enrichment procedure may also be employed. Inoculate a portion of the specimen into CMB, or into cycloserine cefoxitin fructose broth (CCFB) which is CCFA medium omitting agar and egg-yolk; O'Farrell et al (1984) recommend including sodium taurocholate 1 g/litre in CCFB for enrichment of non-faecal specimens. Incubate anaerobically for 24 h at 37°C and subculture on fresh CCFA as above.

5. Examine plates after anaerobic incubation for 24 h at 37°C. The CCFA medium is highly selective, but an occasional yeast or lactobacillus may grow. On CCFA medium, or on CCFA in which the egg-yolk has been replaced by blood, *C. difficile* colonies show a yellow/green fluorescence in long wave (365 nm) UV light. Reincubate plates for a further 24 h before discarding. The use of an anaerobic cabinet for incubation of CCFA plates is particularly valuable since it allows reincubation of plates after inspection at 24 h without exposure to air.

6. Subculture any suspect colony to BA and incubate anaerobically without delay. Subculture to PPYG medium; after 24 h at 37°C prepare a specimen from the PPYG culture for GC (see Ch. 36, *Methods*). A more rapid GC confirmation of this organism is done by demonstrating the presence of *p*-cresol and caproic acid in cores of agar beneath suspect colonies on CCFA medium supplemented with *p*-hydroxyphenyl acetic acid and DL nor-leucine (Phillips & Rogers 1981).

7. If GC is not available, do a range of biochemical tests as indicated in Tables 36.1 and 37.4.

8. Consider a biotyping or serotyping approach if this is available for the investigation of any suspected *C. difficile* outbreak. Isolates may also be assessed for cytotoxin production.

CLOSTRIDIUM BOTULINUM

Botulism is a rare but often fatal intoxication of man and animals. *C. botulinum* spores are widely distributed in nature and botulism is the

* Refer to *Methods* at end of this chapter.

result of ingesting food in which *C. botulinum* has multiplied and produced toxin. The disease is characterized by pronounced toxic effects on the peripheral cholinergic nervous system in which the release of acetylcholine at peripheral synapses is irreversibly blocked. After ingestion of the toxin, within 24 h or less, there is progressive paralysis, often initially affecting oculomotor and oropharyngeal muscles. Death usually results from paralysis of the muscles of respiration. *C. botulinum* types A–G are differentiated on the basis of their antigenically distinct but pharmacologically identical toxins. Types A, B and E are most frequently associated with human cases of botulism, but all types can cause disease in man.

Infant botulism is a recently recognized disease in which ingested *C. botulinum* spores multiply in the immature infant gut and produce toxin which is absorbed and produces a spectrum of disease ranging from a mild failure to thrive to paralysis and a sudden infant death syndrome (Arnon 1980).

Wound botulism is an extremely rare disease arising from contamination of a wound or devitalized tissue with *C. botulinum*. The toxin gives rise to neurological symptoms after an incubation period of 4–14 days.

Morphology and staining

Straight or slightly curved rods, 4 × 1 μm but ranging from 2–10 × 0.5–2 μm, arranged singly or less commonly in pairs or short chains. Spores oval, subterminal and may slightly distend the cell; some highly toxigenic strains produce few spores. Motile with peritrichous flagella. Gram positive, but become Gram negative when sporulating.

Cultural characters

Strict anaerobe. Grows well on most rich laboratory media. The different toxin types also differ in their cultural characters and there is some variability within each toxin type. Smith (1977) divided the species into four groups (I–IV, see Table 38.1) based on cultural characters. Optimum temperature 30–37°C, depending on strain. Colonies on solid media after incubation for 48 h are irregularly circular, large (3 mm), smooth, greyish, translucent, with a fibrillar edge that often becomes spreading. Most strains are haemolytic on horse blood agar. Proteolytic strains (A, B, F and G) produce partial clearing on heated blood agar. All types except G produce restricted opalescence and a pearly lipolytic effect on egg-yolk agar (Willis 1977). Recent experience with selective egg-yolk agar containing cycloserine, sulphamethoxazole and trimethoprim indicates that this is very useful for the confirmation of botulism, especially infant botulism (Hatheway & McCroskey 1981).

Biochemical reactions

The groups shown in Table 38.1 show different biochemical reactions. Group I strains ferment glucose and fructose and produce variable or negative results with other sugars; group II strains ferment fructose, glucose, mannose and sucrose; group III strains ferment glucose, mannose and ribose; and group IV (type G) is asaccharolytic. The proteolytic activities of the various types are summarized in Table 38.1. Most strains are indole negative. Tables 37.2, 37.4 and 37.5 indicate other biochemical reactions of interest.

Table 38.1 Cultural groups of *C. botulinum* (after Smith 1977).

Characters of organism	Cultural group			
	I	II	III	IV
Fermentation of glucose	+	+	+	−
Digestion of coagulated protein	+	−	−[a]	+
Hydrolysis of gelatin	+	+	+	+
Major metabolic products[b]	A, P, iB, B, iV	A, B	A, P, B	A, iB, B, iV
Toxin types	A, B, F	B, E, F	C_1, C_2, D	G

[a] Some strains show weak proteolysis.

[b] Volatile fatty acids detected by gas chromatography (GC): A, acetic; P, propionic; iB, iso-butyric; B, butyric; iV, iso-valeric.

C. sporogenes is biochemically indistinguishable from *C. botulinum* group I, and *C. novyi* type A is biochemically indistinguishable from *C. botulinum* group III; differentiation of these organisms depends on specific toxin testing (Smith 1977).

Toxins

There are eight serologically distinct types – A, B, C_1, C_2, D, E, F and G. Slight cross-reactions occur between C_1, C_2 and D, and between E and F. Most but not all of these toxins are synthesized as non-toxic or slightly toxic protoxins. Type A strains and some of the proteolytic type B and F strains produce a protease that activates the toxin; most type E strains and the non-proteolytic B and F strains require an exogenous protease to activate the toxin. Some strains of types C and E produce fully active toxins. All toxins are thermolabile and are inactivated by formaldehyde. Specific phages are involved in the production of toxin by *C. botulinum* types C and D, which can be interconverted; similarly, *C. botulinum* type C and *C. novyi* type A can be interconverted by phages (Eklund & Poysky 1981).

Spores

C. botulinum spores are among the more resistant bacterial spores. There is a descending gradient of heat resistance from the most resistant spores of group I to the less resistant spores of group III. Spores of type A strains can resist boiling at 100°C for some hours. Spores of type E strains are said to be relatively heat sensitive, but this may be misleading; the heated spores of some type E strains do not germinate until they have been treated with lysozyme or a proteinase. Spores of *C. botulinum* are also resistant to radiation, UV light, alcohols, and phenolic and quaternary ammonium compounds. They are relatively susceptible to hypochlorite, ethylene oxide and formaldehyde. The reader is referred to a review by Smith (1977, Ch. 3).

Typing methods

The specific toxin type is determined by toxin/antitoxin neutralization tests in mice. Fluid specimens are mixed with monovalent type-specific antitoxins (Institut Pasteur, 28 Rue du Docteur Roux, 75724 Paris) and injected intraperitoneally into mice. Controls include heated and unheated specimens without antitoxins.

The four groups of *C. botulinum* determined on the basis of cultural and biochemical tests appear to possess group-specific surface antigens. Immunofluorescence procedures with fluorescein-labelled antibodies (FA) can achieve partial typing, but preparations have varied greatly in their usefulness (see Walker & Batty 1964). Three FA reagents available from Wellcome are: anti-type A with activity against A, B and F; anti-type C which reacts with C and D; and anti-type E which reacts specifically with type E. A polyvalent anti-ABF FA reagent is available in the USA. Note that some immunofluorescence reagents cross-react with *C. sporogenes*.

Animal pathogenicity

Intraperitoneal inoculation of culture fluid or culture supernate into mice gives rise to respiratory difficulty in the animals within a few hours. Paralysis of abdominal muscles gives a wasp-waist appearance. A flaccid or generalized paralysis develops; death usually follows within 18–24 h but may take up to 4 days. The internal organs are congested, and thromboses and haemorrhages are seen at autopsy.

Laboratory diagnosis of botulism

Note that *C. botulinum* and materials that may be contaminated with *C. botulinum* must be handled under appropriate containment conditions (Ch. 15). Guidelines for submission of specimens to the Food Hygiene Laboratory, CPHL, London, should be consulted (Report 1979).

Specimens to be investigated may include faeces, food, vomit, gastric fluid, serum, environmental samples and occasionally wound exudate or tissue. The most conclusive proof of botulism is the detection and specific neutralization of toxin in the patient's serum by toxin/antitoxin tests in mice. As direct toxin detection is not always possible, other investiga-

tions are added as a routine, and these include culture and identification of the organism from specimens with subsequent detection of toxigenicity. Sometimes the organism may be detected by direct immunofluorescence.

Detection of toxin in serum, food, vomit, faeces or supernates

The following procedure is adapted from that described by Willis (1977).

1. Prepare liquid extracts of solid specimens by homogenization with an equal volume of sterile saline and subsequent centrifugation.

2. As some toxins are activated by trypsin, treat half of the extract with trypsin solution (1% Difco Trypsin 1:250 in distilled water) in the proportions of 9 parts extract to 1 part trypsin solution, held for 1 h at 37°C. The trypsin-treated and untreated samples are again divided into two; heat one portion of each sample to 100°C for 10 min to provide inactivated controls. *Note*: when testing for toxin in serum, trypsin treatment is unnecessary.

3. Add penicillin to each of the four specimens to give a final concentration of 200 units/ml.

4. Challenge three pairs of mice by intraperitoneal inoculation of the untrypsinized material as follows. Protect the first pair with intraperitoneal polyvalent botulinum antitoxin (A, B and E; Institut Pasteur) and challenge with 0.5 ml doses of the unheated material. Challenge the second pair, unprotected, in the same way. Challenge the third pair, also unprotected, with 0.5 ml doses of the heated material.

5. Challenge a further three pairs of mice as above but with the trypsinized material.

Five results are possible: (a) Unprotected mice receiving unheated sample die, while protected mice, and those receiving heated sample survive; this confirms the presence of botulinum toxin. (b) Increased toxicity of some toxins (e.g. type E) by trypsinization may cause death of protected animals – check by diluting sample. (c) If none of the protected animals survives, either toxin C, D or F, or a potent toxin of the other types is present. Repeat with mice protected with C, D and F antisera and use diluted samples. Tetanus intoxication is another possibility – use mice protected with tetanus antitoxin. (d) All animals die including those receiving heated sample – indicates a non-specific toxic substance. (e) All survive – indicates the absence of botulinum toxin.

Culture of C. botulinum

Direct detection of the organism in the specimen by a fluorescent antibody (FA) procedure is possible, but culture is often necessary and this is generally regarded as technically difficult. See Willis (1977) and Hatheway & McCroskey (1981) for a fuller account.

1. If Gram-positive sporing rods are observed in a specimen, try direct plating, especially in a case of infant botulism. Egg-yolk agar (EYA) has been the medium of choice; a new selective EYA, *C. botulinum* isolation (CBI) medium* will probably supersede it.

2. Attempt enrichment as follows after adding sterile saline to the sample and homogenizing it if necessary. Divide the sample into two. Add an equal volume of absolute ethanol to one half, and leave the other portion untreated. Take 1 ml volumes of ethanol-treated and untreated samples and add these to two sets of three tubes of CMB. Hold one of each set in an 80°C water bath for 10 min, and another pair for 20 min; the final pair is left unheated. This procedure selects for resistant spore-formers but also allows sensitive spores to survive in the untreated cultures.

3. Incubate these cultures anaerobically at 30°C and screen at intervals for toxin production by mouse tests as above; FA tests may be of value as a first screen (see *Methods*, Ch. 37). If no toxin is detected after incubation for 3–5 days, *C. botulinum* should be considered not present.

4. Plate any toxin-positive broth culture on to EYA and CBI medium. An anaerobic chamber should be used, especially if types C or D are suspected. Incubate plates at 30°C for 36–48 h. Colonies showing restricted opalescence and a pearly layer (lipase activity) should be subcultured to CMB and tested for specific toxin in due course. If toxin was detected at the enrichment stage and no opalescence or pearly layer effect is observed on the EYA subcultures, type G may be suspected. FA procedures may

again be useful at this stage for rapid confirmation.

METHODS

Cycloserine cefoxitin fructose agar (CCFA) (George et al 1979)

Basal medium

Proteose peptone No. 2 (Difco)	40 g
Na_2HPO_4	5 g
KH_2PO_4	1 g
NaCl	2 g
Anhydrous $MgSO_4$	0.1 g
Fructose	6 g
Agar	20 g
1% ethanolic solution of neutral red	3 ml
Distilled water	1000 ml

Method. Sterilize basal medium at 121°C for 15 min in 100 ml portions. Add to 100 ml melted base at 50°C: D-cycloserine (Sigma) to a final concentration of 500 μg/ml, cefoxitin (MSD) to a final concentration of 16 μg/ml, and 5 ml of 50% Egg-yolk suspension (Oxoid). Alternatively, the egg-yolk may be replaced with 5 ml of whole horse blood to make CCFA with blood.

Note that the concentration of antibiotics can be halved when CCFA is used with an alcohol procedure to select for spores of *C. difficile* (see text).

Assay for C. difficile cytotoxin

The specimen may be a liquid stool, or the homogenate of a solid stool in physiological saline, or the supernate of a 5 day culture of *C. difficile* in BHI/PP broth (Brain heart infusion (Oxoid) 3.7% w/v and Proteose peptone (Difco) 1% w/v). The supernate may be filter-sterilized, but this is unnecessary.

Prepare four-fold serial dilutions of the test liquid in physiological saline and add 10 μl volumes to 90 μl of Eagle's modified maintenance medium (Gibco: Flow) containing penicillin (200 units/ml), streptomycin (200 μg/ml), gentamicin (10 μg/ml) and amphotericin B (20 μg/ml), over monolayers of human embryo fibroblasts (or a wide range of other cell lines) in Microtitre plates (see Ch. 50). The highest dilution of the test fluid that produces complete rounding of more than 50% of the cells after incubation for 24 h at 37°C gives the cytotoxin titre. Neutralization is performed by adding 10 μl of a 25-fold dilution of *C. sordelli* antitoxin (Wellcome) to a duplicate series of wells. A positive control, either a known positive stool extract or a purified *C. difficile* cytotoxin, should be included; we have found no loss in potency if the toxin control is frozen and thawed several times.

Clostridium botulinum isolation (CBI) medium (Dezfulian et al 1981)

Antibacterial supplements. Prepare the following solutions and sterilize by filtration: D-cycloserine, 1% solution in water; sulphamethoxazole, 1.9% solution in water – add 10% NaOH till dissolved; trimethoprim, 0.1% solution in water – add 0.05 mol/litre HCl till dissolved.

Basal medium

Trypticase peptone (BBL)	40 g
Na_2HPO_4	5 g
NaCl	2 g
$MgSO_4$ 5% aqueous solution	0.2 ml
D-glucose	2 g
Yeast extract (Difco)	5 g
Agar	20 g
Distilled water	900 ml

Method. Adjust to pH 7.4 and autoclave at 121°C for 15 min; cool to 55°C. Add 100 ml of Egg-yolk suspension (50% in saline; Difco) and antibacterial supplements prepared as above: D-cycloserine, 25 ml (250 mg); sulphamethoxazole, 4 ml (76 mg); and trimethoprim, 4 ml (4 mg).

Store plates in an anaerobic chamber for at least 4 h before use.

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Methods for the Immunological Analysis of Anaerobes

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1. Abstract

The reasons why anaerobes are studied by immunological methods include the development of techniques for the diagnosis of disease, understanding pathogenic mechanisms, epidemiological investigations, development of vaccines and immunotherapeutic agents, in various biotechnological applications and in taxonomic studies. The antigens of anaerobic bacteria are similar to those of other Gram-positive and Gram-negative bacteria and these are described. Two methods are given for the preparation of conventional polyclonal antiserum. The various immunological methods that can be used for investigating anaerobes are listed and several discounted as being of little use today. Modern methods such as crossed immunoelectrophoresis, ELISA and immunoblotting are described in some detail and the advantages and disadvantages of each are described.

2. Introduction

It is perhaps those anaerobic bacteria of clinical importance that are most widely known and this paper will tend to concentrate on these. However, before beginning to describe the immunological methods that can be exploited, we should question the reasons for doing so. This is not an active area of research, perhaps because the work is fraught with difficulties and so few anaerobic infections have a simple one organism–one disease relationship. Most anaerobic infections have complex, poorly understood aetiologies. This paper will begin by explaining the reasons why we should use immunological methods to study

anaerobes. This will be followed by a description of the antigens that can be exploited and the methods for producing conventional polyclonal antiserum. Finally, the immunological methods currently available will be discussed and a selection of the most useful will be described.

3. Why Use Immunological Methods to Study Anaerobes?

The most obvious medical reason that one may first think of is serology: the detection and measurement of specific antibodies in serum specimens. Is this a useful approach? There are relatively few anaerobic infections with a well established single pathogen and these include some of the clostridial infections of man and animals. It may be in chronic infections, or deep abscesses, or infections where the pathogen cannot be cultured, or infections with complex microflora consisting of both commensals and pathogens, the latter also perhaps originating from normal flora, that serological tests may be most useful. In acute infections it is unlikely that an antibody response will be detectable early enough and it is better to isolate and identify the organism.

An alternative approach in acute infections is to detect free antigen or, perhaps more likely, to detect antigen bound to antibody, i.e. immune complexes. These should both be detectable very early in the course of the infection and their detection should be an accurate and sensitive way of making a diagnosis, even if the infecting organism is non-viable because of the action of the immune response or antibiotics, or it cannot be cultured. It is of course possible to detect tetanus, botulinum and *Clostridium difficile* toxins by toxin–antitoxin neutralization tests, but in the future it may be possible to detect specific antigens in periodontal disease: perhaps, for example, spirochaete antigens or *Bacteroides gingivalis* antigens. In vaginosis the detection of *Mobiluncus* spp. antigens may be useful and it may be possible to detect specific bacteroides antigens in serum or cerebral spinal fluid. For more details on the detection and clinical significance of immune complexes see ref. 1.

In epidemiology a knowledge of the antigenic make up of an organism is of obvious use. The candidate antigen tests range from traditional O and K serotyping and toxin typing to immunochemical fingerprinting. It is possible to serotype *B. fragilis* by use of O antigens but the clinical significance is not yet apparent (2). Toxin typing is important for identifying *C. perfringens* and for the investigation of botulism. Analysis of surface antigens of *C. difficile* has proved most useful in epidemiological investigations of outbreaks of antibiotic-associated pseudomembranous colitis and diarrhoea (3).

It is in studies of the pathogenic mechanisms of anaerobic infections that it is most useful to have immunological probes and markers to investigate such virulence factors as capsules, lipopolysaccharides, fimbriae, surface proteins and toxins, and to be able to observe the effect of neutralization of these components with specific antibodies or to observe their effects on complement

action and phagocytosis. The knowledge gained from an understanding of the pathogenic mechanisms can be made use of in the prevention and treatment of infections.

Examples of active immunization are well known for tetanus and botulism, and several other clostridial infections of animals. The value of passive immunization for the two former conditions, along with gangrene, is debatable. Might

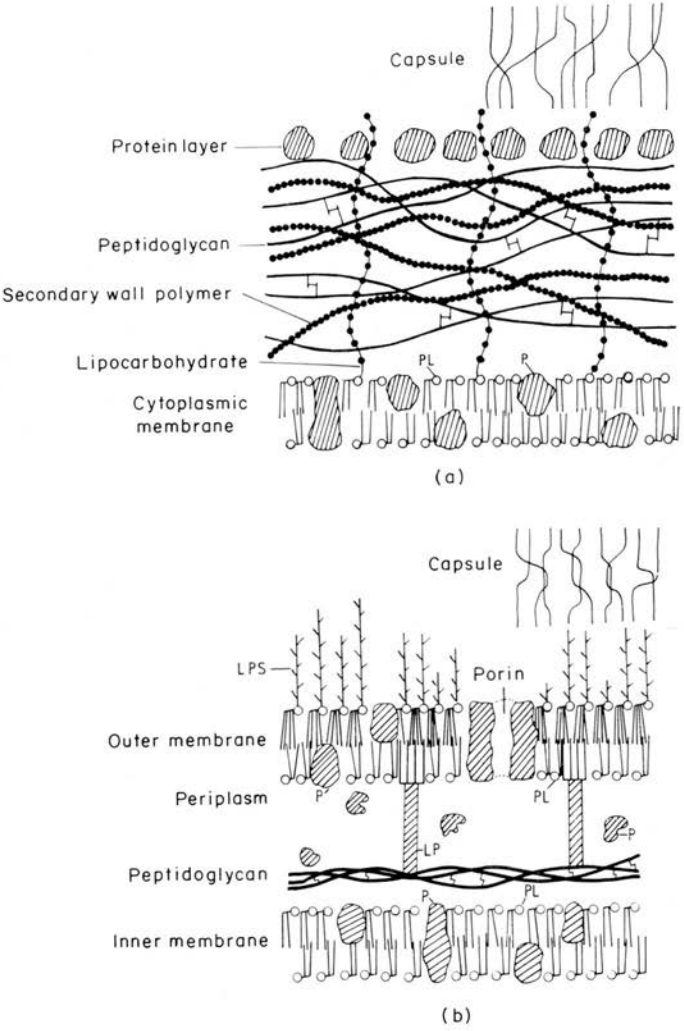

Figure 1. Diagrammatic representation of the cell envelope of (a) a Gram-positive and (b) a Gram-negative bacterium. LP, lipoprotein; LPS, lipopolysaccharide; P, protein; PL, phospholipid. (Figure reproduced from ref. 5.)

Table 1. Antigens that can be exploited.

Gram-positive bacteria
Secondary wall carbohydrate (teichoic acid)
Membrane lipocarbohydrate (lipoteichoic acid)
Wall proteins (regular arrays)
Gram-negative bacteria
Lipopolysaccharide
Outer membrane proteins
Lipoproteins
Fimbriae/pili
Either Gram-positive or Gram-negative
Capsules
Flagella
Extracellular products (exotoxins)

immunization to prevent adhesion be useful for preventing any anaerobic infection? The whole subject of immunotherapy is a new and exciting area of research and it is possible that some anaerobic infections may be controlled in this manner.

In non-medical areas such as in biotechnology, anaerobes may produce commercially useful products. Antibodies, whether polyclonal or monoclonal, might be used as probes for cloned gene products and as immunosorbents in product purification. Finally, in taxonomy, antibodies are useful tools with which to investigate relationships between organisms, and they have been used widely.

4. Antigens of Anaerobes

In all the work reported on the morphology, structure and chemistry of their cell surfaces, anaerobes appear similar to the aerobes and facultative anaerobes and can be thought of as typical Gram-positive and Gram-negative bacteria. Diagrammatic representations of the surfaces are shown in Figure 1 and the major antigens that can be exploited in any immunological investigation are listed in Table 1. It should be noted that the production and fine structure of most if not all antigens is very dependent on the conditions in which the organism is cultured. The rich complex media employed for laboratory isolation and identification are likely to produce antigens with a significantly different chemistry from those grown *in vivo* and culture methods should attempt to mimic the conditions *in vivo* as closely as possible. Methods for the isolation and purification of antigens are described in refs 4–5.

5. Antisera

In this paper only two methods are given for the production of polyclonal antiserum. They have proved successful for many clostridia and bacteroides.

Details and recommendations for the preparation of monoclonal antiserum are given in Chapter 18 by J. H. Reid.

5.1. Method for whole bacteria

Inject 10^9 bacteria in 1 ml buffered saline into the marginal ear vein of a rabbit on days 1, 2, 3, 8, 9, 10 and 22. Test bleed on day 29 and exsanguinate by cardiac puncture if sufficient antibody is present. A further boost is unlikely to increase the levels more than two-fold. For *Bacteroides* spp., washed live cells can be injected directly. For *Fusobacterium* spp., which tend to produce a more toxic lipopolysaccharide than bacteroides, and for certain clostridia that produce potent exotoxins the number of cells should be reduced to 10^6–10^7 for the first three injections and increased stepwise to 10^9. Cells from these genera should also be killed by exposing a thin film of cell suspension in a glass petri dish to short-wave ultraviolet light until no viable cells can be cultured. This has to be checked by experimentation for each ultraviolet lamp.

5.2. Method for subcellular or purified antigens

Prepare injections containing 0.1–2.0 mg antigen (toxoided by treating with 0.5% formaldehyde for 18 h) in a total of 1 ml Freund's complete antigen (1 : 1 aqueous phase : adjuvant). Inject subcutaneously in several sites on the back of a rabbit. Repeat after 2–4 weeks with incomplete Freund's adjuvant. Test bleed after a further 1–2 weeks and boost if necessary.

Both of these methods can be used in the mouse if they are suitably scaled down. Intravenous injections of 0.1 ml can be readily administered through the tail vein and weights of antigen should be reduced 10- to 100-fold.

6. Methods for Immunological Analysis

The methods that have been or might be used for immunological analysis of anaerobes are listed in Table 2. Those not in italic letters can probably be discounted as they are out of date, insensitive, time consuming or of poor specificity and have been largely superseded by more modern methods.

6.1. Enzyme-linked immunosorbent assay (ELISA)

This is probably the most useful and widely used immunoassay available at present. It can be used for the detection of both antigen and antibody; it is very sensitive and simple to use; in the standard 96-well format large numbers of samples can be analysed; and it is suitable for automation. Monovalent antigens can also be detected as long as the binding to the plate does not block the epitope. The greatest disadvantage of ELISA is that if a mixture of antigens

Table 2. Immunological methods.

Gel diffusion
Immunoelectrophoresis
Counter-current immunoelectrophoresis
Crossed immunoelectrophoresis
Rocket immunoelectrophoresis
ELISA
Immunoblotting
Immunofluorescence
Radioimmunoprecipitation
Agglutination
Coagglutination
Latex agglutination
Toxin neutralization
Immunoelectronmicroscopy
Complement fixation
Indirect haemagglutination

Italics, present-day methods of importance; Roman, out-dated methods.

is used to coat the plate it is not possible to know which antibodies are binding without performing an inhibition assay. More details of the applications of ELISA can be found in refs 6–7.

6.2. Crossed immunoelectrophoresis (CIE)

Crossed immunoelectrophoresis (CIE) and its one-dimensional relative, rocket immunoelectrophoresis, have been largely superseded by immunoblotting and the much more difficult technique of radioimmunoprecipitation. There are, however, several applications for CIE. This technique has the advantage that the antigens are not denatured and the pore size of the agarose support matrix allows extremely large antigens such as capsular polysaccharides to be analysed. Rocket immunoelectrophoresis is a valuable technique for screening large numbers of specimens or column fractions. Both of these techniques are quantitative and sensitive but only precipitating antigens are detected and it is rather expensive in antiserum. The methods for the techniques have been described in detail elsewhere (8–10).

6.3. Immunoblotting (Western blotting)

This is a powerful modern technique that can resolve complex antigen mixtures. It is fairly simple to perform and extremely sensitive. Its major drawbacks are that the antigens must first be run on a denaturing polyacrylamide gel which may result in the loss of epitopes, especially if monoclonal antibodies are used

as probes, and antigens with a molecular mass of more than a few hundred kilodaltons cannot enter the gel: it is therefore of little use in the analysis of large molecular mass capsular polysaccharides. The methodology and some applications of immunoblotting can be found in refs 11–13.

7. Conclusions

In conclusion, modern immunological methods have yet to be used widely in the study of anaerobic bacteria. In our attempts to understand the complex pathogenic mechanisms of anaerobic infections they have a major role to play. Recently they have proved useful in epidemiological studies. It is perhaps in rapid diagnostic techniques that a future role lies. Once we establish causal relationships between specific pathogens and infection can we begin to develop methods for the early detection of specific antigen. Candidate infections include periodontal disease, infections of the female genital tract and deep abscesses. It should not be forgotten, however, that one of the oldest and most useful of diagnostic techniques in anaerobic bacteriology—the Nagler reaction—is a simple demonstration of neutralization of phospholipase with a specific antiserum.

8. Acknowledgements

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6

Immunochemical methods

I. R. POXTON

1. Introduction

As in any branch of modern bacteriology, immunological techniques are used for a vast and increasing number of applications. The applications in the past were mainly in medical microbiology, especially in the diagnosis of disease, in epidemiological typing, and in the fundamental investigation of pathogenic mechanism. In the future this trend is likely to continue, but the exploitation of immunological methods for the detection and quantitation of any bacterial component or product is likely to increase. In anaerobic bacteriology, however, it is by no means an area of great activity. Many workers have been disillusioned by the complexity of the subject, and it is true to say that much of the past work has been greatly compromised by a lack of appreciation of the complexity of the antigenic composition of anaerobes. They are no more complex than aerobes but, especially for the *Bacteroides* spp., there are no traditional serotyping schemes available as there were for the enterobacteria, and attempts at extrapolating from enterobacterial serology have been largely unsuccessful. Apart from certain clostridial infections there is little evidence of a one organism one disease relationship and again this has confused the unwary.

This chapter will give details of the immunological methods that will be of use in the investigation of most problems likely to be encountered in anaerobic bacteriology. Some examples of applications will be given where relevant and the many problems and pitfalls highlighted.

2. Historical applications of immunological methods

Before beginning to describe the techniques and their applications, it is perhaps worthwhile reviewing, and reminding the reader, of the immunological methods used in the past. It is in clostridial identification that they have been most useful. From the universally used Nagler reaction for the specific identification of *C. perfringens* on egg-yolk agar (see Chapter 3) to the definitive identification of *C. tetani* and *C. botulinum* by toxin neutralization tests in mice (see Chapter 9), the use of specific antibody neutralization tests has been wide. Immunofluorescent antibody methods have been developed for certain clostridia. Capsular

typing based on agglutination tests has been used for investigation of *C. perfringens* food poisoning strains. Several attempts have been made to develop a somatic typing scheme for the *Bacteroides fragilis* group, but these have been largely unsuccessful and their relevance has been questioned.

3. Antigens of anaerobic bacteria

Anaerobic bacteria can be considered as typical Gram-positive and Gram-negative bacteria (*Figure 1*). As far as we know there are no fundamental differences in structure between aerobes and anaerobes. The range of antigens that can be exploited include both cell-surface components and extracellular products (exotoxins and enzymes).

These antigens include

in Gram-positive bacteria

- secondary wall carbohydrate (teichoic acid)
- membrane carbohydrate (lipoteichoic acid)
- surface proteins (regular arrays and fibrillar proteins)

in Gram-negative bacteria

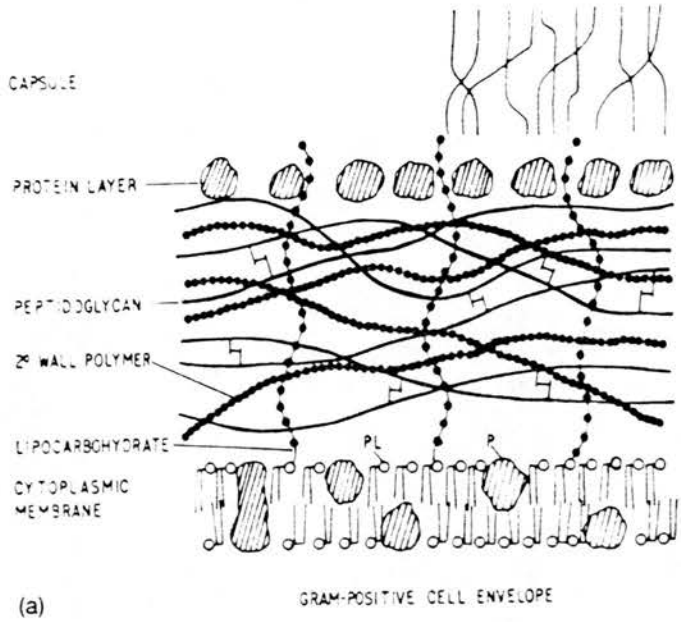
- lipopolysaccharide
- 'common antigens' (equivalent to the enterobacterial common antigen)
- outer membrane proteins
- lipoproteins
- fimbriae pili

in both

- capsular and other exopolysaccharides
- peptidoglycan
- flagella
- extracellular products (exotoxins and exoenzymes)

4. Preparation of some relevant antigens

The preparation of all the antigens just listed cannot be covered in this chapter. Only those considered most important and relevant to anaerobes will be described here. The investigator who requires more detailed information on the preparation and characterization of bacterial antigens is referred to the book by Hancock and Poxton (1). The extraction of antigens from the bacterial cell usually begins with either the whole cell where extractants such as detergents or solvents are employed or, alternatively, the cell may first be fractionated into



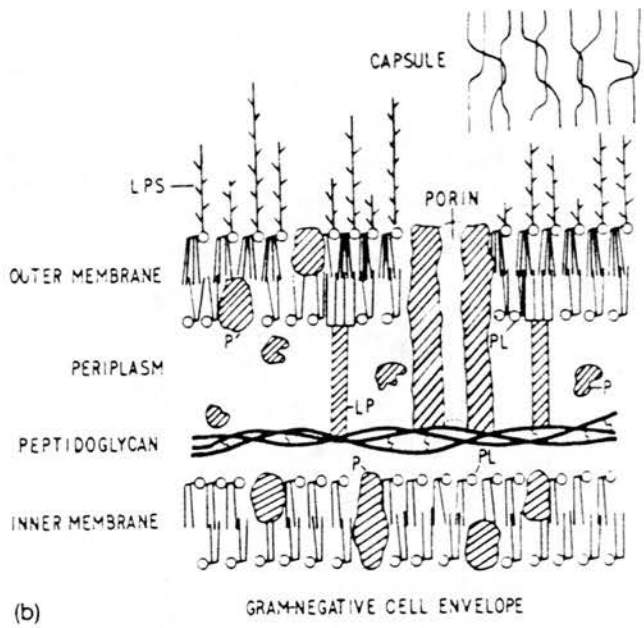


Figure 1. The cell envelopes of (a) Gram-positive and (b) Gram-negative bacteria. PL, phospholipid; LPS, lipopolysaccharide; P, protein; LP, Braun's lipoprotein.

subcellular components such as cell walls, cell envelopes, or membranes, and then subsequent extraction methods are employed on these fractions. For this latter approach, cell breakage is necessary and is most important for Gram-positive bacteria.

4.1 Cell breakage

Two methods will be described: (a) the French Press; and (b) sonication.

4.1.1 The French Press

The French Press (manufactured by Aminco, Silver Springs, Maryland, USA) is a convenient method for breaking all types of bacteria. It has the advantage that the wall/envelope remains largely intact and is not highly fragmented. The bacteria are made into a suspension in a physiological buffer containing 1 mM magnesium, and the cell density can range widely in concentration from very dilute to 50% (wet w/v). The suspension (5–40 ml) is poured into the pre-cooled pressure cell and is assembled in the hydraulic press. Pressures in the range of 10^7 Pa are required for breakage. Generally, Gram-negative rods break most easily, while Gram-positive cocci are the most difficult, and may require several passes through the cell for complete breakage. The efficiency of breakage must be monitored by microscopy and any unbroken cells are removed by two cycles of low-speed centrifugation (5000 *g* for 10 min). In very dense suspensions the viscosity may rise alarmingly after breakage because of the release of nucleic acids. If this is a problem 10–20 μg of DNase and RNase can be added prior to passage through the cell, and incubation at room temperature for 15 min or so after breakage is usually sufficient to reduce viscosity.

4.1.2 Sonication

This technique is usually more widely available than the French Press, but suffers from the disadvantages that only small volumes (usually 10 ml or less) are handled. Heating is a problem and cooling in ice ethanol is necessary. Some bacteria, especially Gram-positive cocci, can be difficult or impossible to break, and, for those bacteria that can be broken, tiny fragments are often produced which make subsequent fractionation difficult. A suspension is made up as described in Section 4.1.1, and a 5–10% (wet w/v) concentration is usually ideal. Depending on the type of bacteria, five 1-min bursts (with 30-sec cooling periods) of peak power from the ultrasonic generator are often sufficient. Monitor breakage by microscopy.

Once cells are broken, it must be recognized that most bacteria possess autolytic enzymes which can greatly modify cellular components in a short time. Subsequent steps must take account of this and procedures must involve inactivation steps, or speed and low temperatures must be employed.

Protocol 1. Wall preparation in Gram-positive bacteria

1. After cell breakage and removal of any unbroken cells, centrifuge the suspension at 40 000 *g* for 20 min. The cell wall/envelope fraction forms a dense layer at the bottom of the tube. For many anaerobic bacteria this layer is often black in colour because of the presence of insoluble sulphides produced in the highly reduced growth conditions.
2. Decant the supernate containing the cell membranes and cell contents and retain for later. Resuspend the wall pellet in water and wash once.
3. Resuspend the pellet in water and add an equal volume of 4% (w/v) sodium dodecyl sulphate (SDS) which has just boiled.
4. Stir the suspension for several hours (conveniently overnight) at room temperature.
5. Wash the suspension free of SDS by at least six cycles of centrifugation (40 000 *g*) with water at *room temperature* (SDS precipitates in the cold). SDS removal can be confirmed by the water not frothing. The cell walls should now appear white.
6. Resuspend in a small volume of water and lyophilize.

After the breakage of Gram-negative bacteria, the envelope fraction, i.e. inner membrane, outer membrane, and associated peptidoglycan layer, is pelleted by centrifugation at forces of 50 000 *g* or greater.

4.2 Preparation of the outer membrane of Gram-negative bacteria

Traditionally, the outer membrane (OM) was prepared from a whole envelope fraction followed by density gradient centrifugation. Although this is probably the most authentic method, an approximation to the method can be made using the detergent sodium *N*-lauroyl sarcosinate (Sarkosyl) which selectively solubilizes the inner membrane from a broken cell suspension, leaving the OM as an insoluble pellet.

Protocol 2. Outer membrane preparation

1. Break cells by either of the methods described in Section 4.1 and remove any unbroken cells.
2. Add Sarkosyl (1 vol of 24% (v/v) solution of Sarkosyl (Sigma: 30% (w/v) solution) to 9 vol. supernate) to give a final concentration of 0.7% by weight.
3. Centrifuge at 50 000 *g* for 1 h to pellet insoluble outer membranes.
4. Resuspend pellet in distilled water and wash as in step 3.

4.3 EDTA extraction of Gram-positive surface antigen

A method that has been used for the extraction of a surface antigen from various clostridia makes use of the chelating agent ethylenediamine tetraacetic acid (EDTA). It appears that a range of surface proteins together with the lipocarbohydrate membrane antigen can be released from clostridia without the lysis of the bacterial cell. This soluble antigen is representative of the whole surface of the bacterium. It has been used in a variety of applications such as in the immunotaxonomy of clostridia (2), especially *C. botulinum* and related species (3), and in the immunological fingerprinting of *C. difficile* (4).

Protocol 3. EDTA extraction of whole bacteria

1. Harvest bacteria from 100 ml broth and wash three times in phosphate-buffered saline (PBS: 50 mM phosphate buffer containing 0.15 M NaCl, pH 7.4) by centrifugation at 10000 *g* for 10 min.
2. Resuspend pellet in 4 ml PBS containing 10 mM EDTA.
3. Incubate at 45°C for 30 min.
4. Remove cells by two cycles of centrifugation as in step 1.
5. The supernate, which will contain in the order of 1 mg/ml protein, can be used without further treatment in ELISA and immunoelectrophoresis assays.

4.4 Secondary cell-wall carbohydrate antigens

The secondary cell-wall carbohydrate antigen or teichoic acid analogue is probably, as in the aerobic Gram-positives, an important grouping antigen in clostridia. It has been investigated only in *C. difficile* (5).

Protocol 4. Preparation of secondary cell-wall carbohydrate

1. Prepare pure cell walls (see *Protocol 1*).
2. Resuspend 1 g in 40 ml of either[a] 0.5 M NaOH or 0.1 M HCl and stir at room temperature for 1 h.
3. Neutralize with the equivalent concentration of HCl or NaOH and remove extracted walls by centrifugation (10000 *g*, 15 min).
4. Dialyse supernate against at least two changes of 5 l of distilled water over 18 h.
5. Concentrate by rotary evaporation and then freeze-dry.

[a] Although both have been used successfully with *C. difficile*, it is well known for aerobes that, depending on the nature of the glycosidic linkages, it is possible that either may depolymerize such polymers. It will be necessary to test each new species individually.

4.5 Lipocarbohydrate antigens

This polymer is analogous to the lipo- or membrane teichoic acid of aerobic Gram-positives, where it is often an important antigen. In aerobes it has been investigated only in *C. difficile* and close relatives (6) and most recently in *C. tyrobutyricum* (7).

Protocol 5. Preparation of lipocarbohydrate

1. The supernate prepared in step 2 of *Protocol 1* is freeze-dried and used as the starting point of the antigen extraction.
2. Dissolve the lyophilized cell supernate in chloroform methanol (2:1) at a concentration of 20 mg/ml and stir for several hours at room temperature. After filtration through Whatman no. 1 paper, re-extract with another volume of chloroform methanol by stirring overnight.
3. Air-dry the filtered, defatted material and then suspend to a concentration of approximately 5% (w/v) in distilled water. Add an equal volume of 80% (w/w) aqueous phenol[a] and stir at room temperature for 1 h.
4. Centrifuge in phenol-resistant tubes at 10000 *g* for 20 min.
5. Remove the upper aqueous phase and dialyse against running water overnight.
6. Remove any insoluble material by centrifugation (10000 *g*, 10 min).
7. For many purposes it may be convenient to interrrupt the preparation at this point and test the material for antigenicity. If a highly purified product is required, it will be necessary to fractionate the material on a column of Sepharose 6B where it will elute in the void volume. Final purification may require immunoadsorbent chromatography (see references 5 and 6 for details).

[a] Caution! Phenol is extremely caustic and toxic; handle with care.

4.6 Lipopolysaccharide

As in all Gram-negative bacteria, lipopolysaccharide (LPS) is present in Gram-negative anaerobes. It is usually considered less endotoxic than its aerobic (*Escherichia coli*) equivalent, but only the clinically important *Bacteroides* and *Fusobacterium* spp. have been investigated. Various serotyping schemes have been developed for the *B. fragilis* group, but their relevance is uncertain. There is confusion as to the relationship between capsular polysaccharide and LPS. There is some debate as to the method for the extraction of LPS (8). The best starting approach when investigating an unknown LPS is to use the proteinase K method developed by Hitchcock and Brown (9).

Protocol 6. Proteinase K method for LPS preparation

1. Harvest bacteria from broth or solid medium, wash once in PBS, and resuspend in PBS to an A_{525} (absorbance of 525 nm) of between 0.5 and 0.6.
2. Centrifuge 1.5-ml volumes in a microcentrifuge at 10000 *g* for 3 min.
3. Suspend pellet in SDS–PAGE sample buffer and heat to 100°C for 5 min.
4. When cool add 25 μl proteinase K (Protease type XI, Sigma) in 10 μl SDS–PAGE sample buffer and incubate at 60°C for 60 min.
5. Analyse 10-μl samples on 14% polyacrylamide gels, omitting the SDS from the stacking and separating gel buffers.
6. Stain gels with silver or transfer to nitrocellulose for immunoblotting (see *Protocol 14*).

Once the chemotype of the LPS (i.e. rough or smooth) is established by the proteinase K method, it is possible to select the classical aqueous phenol method for the preparation of smooth LPS or the phenol/chloroform/petroleum method for rough LPS (see reference 1 for methods). A combination of these methods has been suggested by Weintraub *et al.* (10), but it should be noted that it is possible that the smooth component of the LPS could inadvertently be discarded (8), especially if the LPS only appeared rough because of the silver stain failing to reveal the 'O' polysaccharide chains: these are sometimes periodate-resistant and only revealed by specific antibody in immunoblots.

4.7 Exopolysaccharides

Capsules are found on a wide range of anaerobes, both Gram-positive and Gram-negative. As described in Section 4.6 there is a certain amount of confusion between the exo- or capsular polysaccharide of *Bacteroides* species and the O antigen of the LPS. It is probably true to say that no definitive method exists for the preparation of exopolysaccharide. In attempting to begin the preparation of the capsule, mechanical methods of shearing off the capsule should be the first approach.

Protocol 7. General method for capsule preparation

1. Harvest bacteria from solid medium by gently scraping with a glass rod and suspend in PBS containing 0.5% formaldehyde.
2. Shear off capsule: gentle stirring to violent agitation in a blender or homogenizer may be required. Monitor by microscopy with indian ink staining.
3. Remove bacteria by centrifugation with forces >10000 *g* because of high viscosity. Monitor removal of cells by microscopy.

Protocol 7. *Continued*

4. Add four volumes of ice-cold acetone to the supernate and allow the capsular material to precipitate overnight at 4°C.
5. Wash precipitate several times in acetone, dissolve in water, and lyophilize.
6. At this stage the material should be checked for antigenicity. It will almost certainly be contaminated with proteins and other carbohydrates, especially LPS in Gram-negative bacteria. The full purification will require often difficult biochemical and immunological separation techniques which are compounded by the high viscosity and high molecular mass of the material.

4.8 Preparation of appendages

Bacterial appendages are well known antigens, but in anaerobes very little is known of fimbriae or pili. Only the preparation of flagella will be covered here. They have been investigated in several clostridia (11, 12).

Protocol 8. Preparation of flagella

1. Harvest motile bacteria from a log phase broth culture (1 l) by centrifugation at 10000 *g* for 15 min. *Gently* resuspend in 1 l of PBS and recentrifuge.
2. Resuspend in 20 ml PBS and homogenize in a rotating blade blender at maximum speed for 1–2 min.
3. Remove bacteria by at least two cycles of centrifugation at 10000 *g* for 15 min.
4. Pellet crude flagella by centrifugation at 100000 *g* for 90 min. The pellet may be extremely difficult to see, but it should be resuspended in a small volume of PBS and monitored by electron microscopy (by shadowing or negative staining).
5. Prepare a caesium chloride solution (1.3 g/ml) by dissolving optical grade CsCl in PBS to give a refractive index of 1.3630 (*ca.* 2.125 g is required). Add this to the pellet of flagella and mix well. Centrifuge at 180000 *g* for 20 h in a swinging bucket rotor.
6. The flagella form a band in the central region of the tube and are removed with a fine-gauged syringe needle.
7. Recover the flagella by diluting the CsCl solution in the removed band in about 10 ml distilled water and centrifuge at 100000 *g* for 1 h.

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5. Production of antisera

Only the preparation of conventional antiserum in rabbits will be described here. The production of monoclonal antibodies is a much more specialized procedure

and requires special facilities. Nevertheless the immunization procedures described here for rabbits can be scaled down 10- or 100-fold for the immunization of mice prior to hybridoma production.

Only two methods are given here for raising conventional polyclonal antiserum—one for whole bacteria and the other for subcellular or soluble antigens. The doses are based on New Zealand White rabbits of 2–2.5 kg. For the smaller Dutch rabbits, which are becoming more common because of cage size regulations, the dose should be scaled down on a weight for weight basis. As the response to antigens can vary greatly from one animal to another it is usual to use more than one animal for raising antisera.

5.1 Whole-bacteria vaccine

For most anaerobes, a washed suspension of live bacteria in PBS is all that is required. All *Bacteroides* spp., and many of the clostridia, permit doses of 10^9 in 1 ml to be injected from day 1. For the more endotoxic *Fusobacterium* spp. and the clostridia which produce potent exotoxins, it is necessary to reduce the dose to 10^6 organisms for the first three injections and irradiate the bacteria (in a thin film in a glass Petri dish) with a lethal dose of ultraviolet light. It is convenient to prepare one batch of antigen for the whole immunization series, storing volumes for each injection in individual tubes and deep freezing.

The procedure is as follows.

(a) Inject 1-ml doses of bacteria into the marginal ear vein on days 1, 2, 3, 8, 9, 10, and 22.

(b) Test bleed from the ear on day 29 and, if antibody is of sufficient strength, exsanguinate the rabbit by cardiac puncture under terminal anaesthesia.

Boosting with more vaccine after day 29 is unlikely to result in a more than twofold increase of titre.

5.2 Subcellular or soluble vaccine

Toxic antigens, such as powerful exotoxins, should be toxoided prior to injection by treatment with 0.5% formaldehyde for 18 h at room temperature.

(a) Prepare vaccines containing 0.1–2.0 mg antigen in 1 ml amounts by mixing equal volumes of the antigen in aqueous solution, suspension and Freund's complete adjuvant. Thorough mixing in a small homogenizer or by passing from one syringe to another through a fine-bore tube is recommended to produce a water-in-oil emulsion. This can be confirmed by placing a small drop on water where it should remain without dispersing quickly.

(b) Inject the vaccine subcutaneously in several sites in the scapular region of the back and massage firmly. Repeat after about 4 weeks using Freund's incomplete adjuvant.

(c) Test bleed after 2 weeks and boost if necessary; then exsanguinate.

The preparation of serum is conveniently accomplished by allowing the blood to clot in a glass vessel overnight at 4°C. If more than one rabbit was used, the serum should only be pooled after checking for an adequate response in each rabbit. After pooling sera should be divided up into convenient volumes and stored deep frozen. Freezing and thawing should be kept to an absolute minimum.

For some applications, such as crossed and rocket immunoelectrophoresis, a much cleaner result can be obtained if the IgG fraction is prepared. Also, if an antibody is to be labelled, it can be done to a much greater specific activity if it is pure. Several methods exist for the preparation of IgG, but perhaps the simplest, and the most suitable for most purposes, is by ammonium sulphate precipitation.

Protocol 9. Preparation of IgG

1. Prepare a saturated solution of ammonium sulphate in water and add 0.67 ml dropwise at the rate of one drop per second to 1.0 ml serum which is stirred gently in an ice bath.
2. Continue stirring for 15 min; then centrifuge the precipitated IgG at 3000 *g* for 15 min.
3. Resuspend the pellet in 1 ml or less of 0.05 M Tris buffer, pH 8.0 and dialyse against the same buffer. Alternatively a 10 kd cut-off ultrafilter may be used.
4. If the IgG is not to be labelled, it is recommended that bovine serum albumin is added to a final concentration of 1%.

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6. Immunological methods

The last two decades or so have seen great advances in immunological techniques. Before this, double gel diffusion, complement fixation tests, and haemagglutination assays were the main techniques available. These have now largely been superseded. In the early 1970s improvements in gel diffusion techniques resulted in the widespread use of two-dimensional or crossed immunoelectrophoresis. This was the first technique to permit resolution of complex mixtures of antigens. It is a relatively difficult technique requiring a certain degree of dexterity; it can only be done with a few samples at a time and was only useful for precipitating antigens. The related rocket immunoelectrophoresis allowed quantitation of multiple samples but did not give good resolution. For most purposes immunoblotting has taken over these techniques. The development of radioimmunoassays opened up the field to automation of immunoassays and multiple specimen handling; however enzyme immunoassays, especially enzyme-linked immunosorbent assays (ELISA), have superseded this technique. For this chapter, precipitation in gel techniques will only be covered briefly and most emphasis will be placed on ELISA, immunoblotting, and immunogold electron microscopy.

6.1 Precipitation in gels

Simple Ouchterlony-type gel diffusion is now largely superseded and will not be described here. Rocket immunoelectrophoresis is probably a more sensitive substitute and is recommended for screening multiple samples containing precipitating antigens. It is quantitative but it has poor resolution for complex antigen mixtures. Crossed immunoelectrophoresis is recommended for complex mixtures where resolution is required. It is only applicable to a relatively small number of samples, but is useful for investigating large-molecular-mass antigens which are impossible to analyse by the more recent immunoblotting technique (see Section 6.3).

Protocol 10. Preparation of electrophoresis buffer and agarose gel

1. Prepare electrophoresis buffer solution 1 by dissolving in 2 l of distilled water
   - Barbitone sodium 26 g
   - Barbitone 4.14 g
2. Prepare solution 2 by dissolving in 2 l distilled water
   - Glycine 112.5 g
   - Tris 90.4 g
3. Mix equal volumes of solutions 1 and 2 and confirm pH to be 8.8. Use this buffer undiluted in buffer reservoirs.
4. Mix together
   - Agarose (low EEO) 1 g
   - Electrophoresis buffer (from step 3) 25 ml
   - Distilled water 75 ml

Heat with constant stirring in a boiling bath until agarose has dissolved. Triton X100 or other detergents can be added to a final concentration of 1% at this stage, if membranous or poorly soluble antigens are being investigated.

5. Cool to 55°C before pouring gels.[a]

[a] It is convenient to prepare in bulk and dispense in suitable volumes for storage.

Protocol 11. Rocket immunoelectrophoresis

Up to 10 samples can be analysed on a 50 mm square of agarose. For more samples a longer length of 50 mm width is required.

1. Cut a 50-mm square of Gelbond® and place it hydrophilic side up on to a supporting glass plate on an absolutely level surface.

Protocol 11. *Continued*

2. Blank off two-thirds of the sheet with a glass plate and cast a layer of agarose (see *Protocol 10*; *ca.* 1.3 ml required).
3. After setting, cut up to 10 3-mm diameter wells in a linear or staggered pattern.
4. For many purposes it is convenient to show identity between antigens and, if this is the case, the wells should be loaded at this stage with 10-μl volumes of antigen and then left for 30 min in the cold to allow antigens to fuse into the agarose. This technique is then referred to as fused rocket immunoelectrophoresis. If showing identity is not a concern then the wells may be loaded after the next step.
5. Gently mix 2.2 ml molten agarose held at about 55°C with 0.5 ml antiserum which may be diluted in 1 : 4-diluted electrophoresis buffer, and pour on to the remaining two-thirds of the Gelbond®.
6. After setting, place the gel on to a horizontal electrophoresis tank with the wells towards the cathode and connect the ends of the agarose to the electrophoresis buffer (see *Protocol 10*) with presoaked filter paper wicks.
7. Apply 12 V/cm for 16 h at 4°C.
8. Press, wash, and stain with Coomassie blue as described in *Protocol 13*.

Protocol 12. Crossed immunoelectrophoresis

1. Prepare the first-dimension gel by pouring 15 ml molten agarose containing 1% Triton X100 (see *Protocol 10*) on to an 80-mm square glass plate.
2. After setting, place plate on the template (see *Figure 2a*) and cut out wells. Fill with 10–15 μl antigen.
3. Place in a horizontal electrophoresis tank with wells towards the cathode. Connect to buffer with filter paper as described in step 6 of *Protocol 11*.
4. Apply 100 V/cm for 1–1.5 h at 4°C.
5. Remove gel from tank and replace on template. Cut out strips and transfer to the edge of a 50-mm square of Gelbond®, making sure the hydrophilic side is uppermost as in *Figure 2b*.
6. Pour the second-dimension antiserum-containing agarose (0.5 ml of antiserum, suitably diluted in 1 : 4-diluted electrophoresis buffer, plus 3 ml agarose at 55°C) against the first-dimension strip.
7. After setting, replace in the electrophoresis tank with the first-dimension strip towards the cathode and connect the edges to the buffer with wicks.
8. Apply 12 V/cm for 16 h at 4°C.
9. Stain as described in *Protocol 13*.

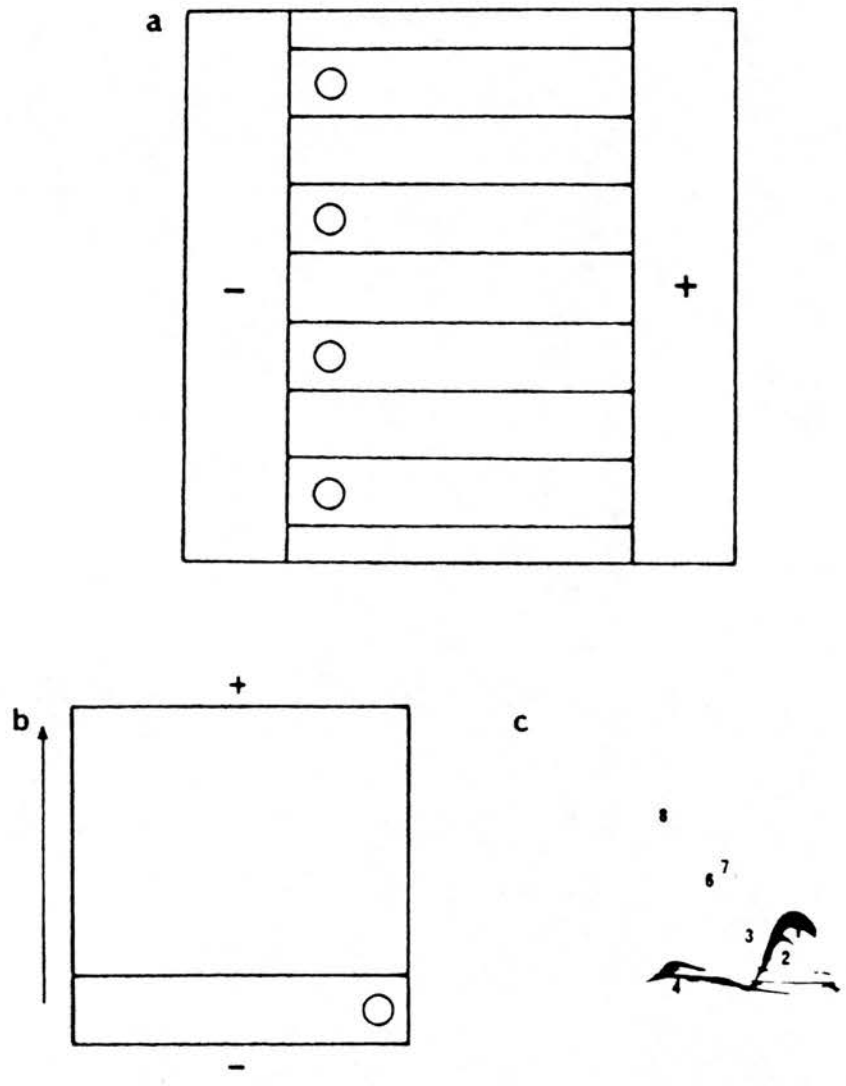

Figure 2. Crossed immunoelectrophoresis (CIE). (a) Template for first dimension—use an 80-mm square of glass; (b) template for second dimension—use a 50-mm square of Gelbond®; (c) an example of CIE: an EDTA extract (see *Protocol 3*) of *Clostridium difficile* (10 µl) run against whole cell antiserum (diluted 1 in 2).

Protocol 13. Staining of gels

1. Place gels on a flat surface and carefully cover with a sheet of Whatman no. 1 paper, avoiding air bubbles.
2. Cover with a stack of blotting paper and apply an even pressure with a weight of about 1 kg (e.g. a glass plate and several books) for 15 min.
3. Remove gels, discard filter paper, and wash in two changes of 0.01 M NaCl for 15 min, followed by a 15-min water wash.
4. Repeat the pressing as in step 2, and then dry the gels in air or with a hot air blower.
5. Place in Coomassie blue stain for 10 min
   - Coomassie blue R250 5 g
   - Ethanol 45 ml
   - Acetic acid 10 ml
   - Water 45 ml
6. Destain with two or three changes of the solution in step 5 from which the dye is omitted.

An example of the use of CIE is shown in *Figure 2c*.

6.2 Enzyme-linked immunosorbent assay (ELISA)

Since its development by Engvall and Perlmann in 1972 (13), this has become the most useful and widely used of all immunological techniques. It has a vast number of applications and many modifications and variations have been made. Both antigen and antibody can be detected and it is extremely sensitive, quantitative, and can readily be automated. The major disadvantage of the technique is that, when it is used for complex mixtures of either antigens or antibodies, it must be remembered that the final colour is proportional to the sum of perhaps many individual antibody antigen reactions and results must be interpreted with caution. For many complex applications it is perhaps worth combining the technique with immunoblotting, which will resolve the individual reactions (see *Protocol 15*). The technique described here is an example of the most commonly used system where antigen is bound to the solid phase (plastic) and specific antibody can be detected. After reaction of antigen and antibody, the complex is detected with a second, antispecies antibody–enzyme conjugate. Its applications range from monoclonal antibody screening to diagnostic serology.

In the latter case it must be remembered that the antigen is all important. If it is impure, minor side-reactions can greatly distort the true picture. This is especially true for anaerobes where, generally, the antigenic makeup of an individual organism is uncertain and the degree of reaction and cross-reaction is

extremely uncertain. Perhaps its safest use is the detection of antibodies to purified toxins. For the detection of antigen the system must be inverted and the antibody solid-phased for use as a capture for the antigen. A second antibody which is enzyme-labelled is then used to create a 'sandwich'. One of the antibodies should be monospecific polyclonal or monoclonal.

Protocol 14. A standard ELISA

1. Make dilutions of antigen in coating buffer (0.05 M sodium carbonate buffer pH 9.6 containing 0.02% sodium azide) and add 100-μl volumes containing 10–100 μg antigen to wells of microtitre plates. Cover and allow to incubate at 37°C for 4 h and then at 4°C overnight.
2. Wash three or four times with 0.9% NaCl containing 0.05% Tween 20.
3. Make dilutions of antibody in antibody/conjugate buffer (0.05 M phosphate buffer, pH 7.4, containing 0.85% NaCl, 0.05% Tween 20, and 0.02% sodium azide) and add to wells. Incubate for up to 4 h at room temperature or 1 h at 37°C.
4. Wash as in step 2.
5. Add 100-μl volumes of suitably diluted anti-first species antibody–enzyme conjugate (dilutions of 1 in 500, to 1 in several thousand are typical) to the wells and continue incubating for several hours (conveniently overnight) at room temperature or an hour or so at 37°C. The most popular enzyme-conjugates are alkaline phosphatase and horseradish peroxidase.
6. Wash as in step 2.
7. Add enzyme substrate diluted according to the manufacturer's instructions and incubate at room temperature for 1 h. The results are conveniently read in a purpose-made spectrophotometer.

6.3 Immunoblotting

Immunoblotting or Western blotting is the technique by which antigens, usually proteins, are separated by polyacrylamide gel electrophoresis and are then electrophoretically transferred to a nitrocellulose or similar membrane, where they are subsequently probed with antibody to locate antigens. Since the transfer method was first developed about 10 years ago by Towbin and colleagues (14) it has become one of the most widely used of immunological techniques with an ever-increasing range of applications and modifications. It has largely superseded the immunoelectrophoresis techniques such as crossed and rocket immunoelectrophoresis. It is used in all areas of immunological research, in diagnostic serology, and is becoming the standard method for the high-resolution detection of any molecule against which an antibody is available. In

anaerobic bacteriology it has been used for immunological fingerprinting of *Clostridium difficile* (6) as well as in numerous other studies for investigating the antigenic composition of anaerobic bacteria.

Protocol 15. Immunoblotting

1. Separate antigens on a conventional polyacrylamide slab gel, and place the unfixed gel on the cathodic side of the transfer apparatus cassette.
2. Carefully overlay with a sheet of nitrocellulose which has been soaked in transfer buffer, avoiding any air bubbles.
3. Assemble transfer cassette and place in a buffer tank containing a high pH buffer (e.g. Tris glycine methanol, pH 8.3: Tris 12 g; glycine 57.68 g; methanol 1 l; water 4 l, adjusting final pH with 1 M NaOH if necessary).
4. Connect to power supply and allow transfer to proceed. An overnight run of 10–12 V, 40 mA is convenient, but faster runs can be made if higher voltages are possible.
5. Remove the membrane and wash for 10 min in Tris-buffered saline (TBS: Tris 4.84 g; NaCl 58.48 g; water 2 l, pH 7.5.
6. Block in 3% gelatin in TBS for 30–45 min.
7. Place in first antibody, suitably diluted in 1% gelatin in TBS, for an hour or more. Dilutions range from as low as 1 in 2 for human serum or monoclonal antibody supernates to 1 in several hundred for hyperimmune rabbit antisera.
8. Wash briefly in distilled water followed by two 10-min washes in TBS with 0.05% Tween 20.
9. Place in second antibody (enzyme-conjugate) diluted in 1% gelatin buffer for at least 1 h. Dilutions of 1 in 500 to 1 in several thousand are usual for most commercial conjugates.
10. Wash as in step 8.
11. Add colour development reagent (which must produce an insoluble coloured product), made up according to the manufacturer's instructions, and allow desired colour to develop.
12. Stop reaction by washing thoroughly in distilled water.

The recently developed semi-dry transfer apparatus with two flat graphite electrodes is gaining in popularity. There are savings in buffer costs and the transfer is more rapid because of the much steeper voltage gradient. An extension to the original concept, where the electrophoretic transfer step is circumvented, is by applying the antigen directly to the membrane as a dot (dot blotting). A

further modification to this technique is the recently described 'line blot' (15) where the lines of antigen are applied with an ink pen point.

The main problems encountered with immunoblotting include: (a) a difficulty in transfer or a lack of binding of the antigen; (b) the antigen losing its antibody-binding capacity after separation and transfer, presumably by denaturation, and the subsequent need for renaturation of the antigen; (c) the problems of high background staining or non-specific binding, and the requirement of blocking agents; (d) a lack of sensitivity when compared to ELISA; (e) the quantitation of the technique; and (f) the inherent problem that the technique is difficult to miniaturize for use in large-scale screening. Many of these problems have been addressed recently with a limited amount of success, and the reader is encouraged to see the recent review by Stott (16).

The final immunological method to be described here (immunogold electron microscopy) is not commonly used, probably because of its complexity, but it is proving invaluable for the detection and location of antigens on intact bacteria. It has been used to great effect with *Bacteroides* by Patrick and colleagues (17).

Protocol 16. Immunogold microscopy

1. Harvest and wash bacteria and resuspend in 0.01 M sodium cacodylate buffer, pH 7.2.
2. Fix in cacodylate buffer containing 2% (w/v) paraformaldehyde and 0.1% glutaraldehyde for 1 h at 4°C.
3. Wash in cacodylate buffer and dehydrate twice in graded alcohols and finally twice in 100% ethanol which has been dried over anhydrous sodium sulphate.
4. Embed in LR (London resin) white resin as follows: mix in 50% resin, 50% ethanol for 1 h at rom temperature. Next mix in 100% resin in open containers in a fume cupboard overnight. After two further changes of resin at 2–3 h intervals, transfer the bacteria to gelatin capsules which have been dried at 60°C for 3 h, fill the remaining space with 100% resin, seal, and polymerize the resin in a 60°C oven for 18 h.
5. Cut thin sections, in an ultramicrotome and place on nickel grids.
6. Perform immunoassay at room temperature by placing sequentially in: (a) 1% bovine serum albumin (BSA) in PBS, pH 7.2 for 15 min; (b) antibody suitably diluted in 0.1% BSA in TBS (see *Protocol 14* but adjust pH to 8.2); (c) hold in TBS BSA prior to washing in a stream of 8 ml TBS coming from a burette; (d) further block in 1% BSA in TBS for 15 min, followed by (e) addition of an appropriate gold conjugate (15–20 nm particles from Janssen Pharmaceuticals); (f) hold in BSA/TBS prior to washing as in (c); and (g) finally rinse in distilled water.
7. Stain with saturated aqueous uranyl acetate in the dark for 30 min.
8. Wash once in distilled water, then in Reynold's lead citrate, and desiccate for

Protocol 16. *Continued*

several minutes before viewing in the electron microscope. To avoid the film of resin disintegrating, anneal the section by playing the electron beam over it at low power before going to high power.

* For negatively stained specimens, bacteria are suspended in distilled water and placed on Formvar-coated nickel grids. The labelling is performed as in step 6 and negatively stained with 1% aqueous ammonium molybdate.

7. Conclusions

The selected methods given in this chapter will be a useful initiation to those wishing to investigate anaerobes by immunological methods. The techniques are now well established in many laboratories, but a thorough awareness of the complexity of the organism must always be borne in mind. A final point, which has not been covered elsewhere in this chapter, cannot be stressed too much; that is the need to recognize that the antigenic composition of any bacterium may be strongly under the influence of its growth environment. This is as true for anaerobes as for any other bacterium. Attempts must always be made to culture the organism in a medium approximating as closely as possible its natural habitat.

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SHORT ARTICLES

GROUPING OF β-HAEMOLYTIC STREPTOCOCCI BY ENZYME-LINKED IMMUNOSORBENT ASSAY

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IDENTIFICATION of β-haemolytic streptococci (BHS) in the diagnostic laboratory has depended traditionally on the use of presumptive tests in the first instance (Wilkinson, 1979). Definitive group identification rests on extraction of group-specific carbohydrate antigens and their reaction with specific antisera in capillary tubes or agar gel; counterimmunoelectrophoresis may also be used (Hill *et al.*, 1975). Other methods of identification include slide agglutination tests (Christensen *et al.*, 1973) and fluorescence staining (Moody, Ellis and Updyke, 1958).

The present study was undertaken to determine whether an enzyme-linked immunosorbent assay (ELISA) could be adapted to provide a reliable system for grouping BHS. Although this type of assay has been employed for detection of protein antigens and antibodies (Engvall and Perlmann, 1972; Russell, Facklam and Edwards, 1976), many workers have been unable to apply it to polysaccharide antigens, which have an overall negative charge that interferes with adsorption to polystyrene surfaces (Dr B. M. Gray, Dept of Pediatrics and Microbiology, University of Alabama in Birmingham, USA). In this paper we report that this difficulty can be overcome by adsorbing whole cells to polystyrene plates. Results of a trial of the ELISA procedure compared with a double-diffusion method in agar gel are presented.

MATERIALS AND METHODS

Streptococci were isolated from patients attending the Royal Infirmary, Edinburgh, or were stock strains held in the laboratory. The 100 strains tested were: 20 group A, 35 group B, 15 group C, 15 group D, and 15 group G. Initially, strains were identified by the following presumptive tests: gram-stained film, haemolysis on blood agar (Brown, 1919), bacitracin sensitivity for group A (Maxted, 1953), the CAMP test for group B (Christie, Atkins and Munch-Petersen, 1944) and the bile-aesculin test for group D (Facklam and Moody, 1970).

Preparation of extracts. The group-specific carbohydrates were extracted as described by Lancefield (1933). Each strain was grown in 50 ml of Todd-Hewitt broth for 18 h at 37°C and the culture was centrifuged at 1000 *g* for 15 min. The packed cells were extracted with 0·4 ml of 0·067M HCl for 10 min. at 100°C, cooled, and neutralised with 0·5M NaOH. After centrifugation, the supernate was stored at 4°C.

Gel-diffusion test. Double diffusion in agar gel was performed by a modification of the Ouchterlony method (Freimer, 1963).

Antisera. Grouping sera for groups A, C, D and G were obtained from Wellcome Reagents Ltd, Hither Green, London SE13 6TL, and used for the ELISA and for the agar double-diffusion tests. Group-B antiserum was raised in rabbits in our own laboratory. Strain O90R (Streptococcus Reference Laboratory, Colindale, London NW9 5HT) was grown in 250 ml of

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Todd-Hewitt broth for 18 h at 37°C, washed twice in 0·9% (w/v) NaCl, killed by suspending in 2% (v/v) formalin for 3 h, and then resuspended in 25 ml of 0·9% NaCl.

An initial intravenous dose of 0·5 ml of vaccine was given and then 1 ml of vaccine was given intravenously twice weekly for 1 month; test bleeds then showed that there had been a strong homologous response by each animal. The rabbits were exsanguinated and the sera, divided into 5-ml amounts, were stored at −20°C until use. Merthiolate (1 in 10 000) was used as a preservative.

Cross-reacting antibodies were absorbed from each of the grouping sera by adding sequentially 10 ml of a 1 in 10 dilution of serum in PBS (0·05M sodium phosphate buffer containing 0·15M sodium chloride, pH 7·4) to packed PBS-washed bacteria from 100 ml of overnight culture of the prototypes representing the heterologous groups (group A strain NCTC8198, group B NCTC9993, group C NCTC8543, group D Colindale strain and group G NCTC9603) and incubating for 30 min. at room temperature.

Preparation of ELISA plates. Each strain was grown in 5 ml of Todd-Hewitt broth incubated aerobically overnight at 37°C. After the cells had been washed once in saline, 0·5 ml of 0·05M sodium carbonate buffer at *p*H 9·6, containing 0·02% (w/v) of sodium azide (NaN_3) was added, and 50 μl of this suspension was dispensed into wells of a disposable flat-bottomed polystyrene microtitration plate (Sterilin Ltd, 43–45 Broad Street, Teddington, Middlesex). The plate was left open and placed in a drying cabinet at 60°C for 1 h to allow cells to adhere to the polystyrene surface.

The ELISA technique was similar to that described by Voller, Bidwell and Bartlett (1976). Each well was washed three times with phosphate-buffered saline containing 0·05% (v/v) Tween 20 (PBST), and shaken dry at the end of each wash; 50 μl of a 1 in 400 dilution of the test serum in PBST with 0·02% (w/v) NaN_3 was then dispensed into the wells and allowed to incubate at 37°C for 1 h. To prevent evaporation, each plate was sealed in aluminium foil. Washing with PBST was repeated three times and the plate was shaken dry. Ten μl of anti-rabbit antibody conjugated with alkaline phosphatase (Miles Laboratories Ltd, Slough, Berks) was added to 5 ml of PBST containing 0·02% NaN_3; 50 μl of this mixture was dispensed into each well.

The plate was covered with aluminium foil and incubated for 1 h at 37°C. Again, wells were washed three times with PBST, shaken dry, and 50 μl of alkaline phosphatase substrate solution, containing p-nitrophenylphosphate (Sigma Chemical Co., St Louis, Missouri, USA) 1 mg/ml of 0·05M carbonate buffer, *p*H 9·8 with 0·001M $MgCl_2$, was added to each well. The plate was then left uncovered at room temperature.

Interpretation of results. Plates were read by eye within 45 min. of the addition of substrate; a colour change from colourless to yellow was read as a positive result. Control wells containing cells + substrate, and substrate alone were always included in the test.

Results

The following observations emerged from this study. (1) There was complete agreement between the results obtained in agar precipitation tests and in the ELISA for the strains tested. (2) The relative specificity of each of the grouping sera in the ELISA was assessed against known prototype strains and only when complete specificity was obtained with these prototypes were the unknown strains tested. It was necessary to do 5–9 absorptions on each of the sera before this degree of specificity was achieved. (3) The commercial antisera could be used at a maximum dilution of only 1 in 8 in the agar double-diffusion technique, whereas a dilution of 1 in 400 produced strong positive results in the ELISA. (4) An increase in incubation times for the separate stages would permit the use of even higher dilutions of serum in the ELISA procedure. However, plates should be read within the specified time to reduce the possibility of residual cross-reacting antibodies affecting the results.

Discussion

Serological grouping of β-haemolytic streptococci in service laboratories is commonly performed by Lancefield's extraction of the carbohydrate antigen followed either by double

diffusion in agar gel or capillary precipitation tests. These methods are not only time consuming but, if tests are not performed with care, false-positive and false-negative results may occur. In recent times, grouping kits have become available commercially; although these are rapid and easily handled, they are without exception expensive, possibly precluding their use in some laboratories.

The ELISA procedure described in this paper is simple, sensitive and quick to perform. It has the same degree of reliability as gel-diffusion methods for grouping β-haemolytic streptococci, and is economical because antisera can be used in high dilutions. However, the fact that there is a certain degree of complexity in the initial standardisation of the assay may make it unattractive to laboratories dealing with only a few samples daily. The role of ELISA for the serological grouping of streptococci may therefore be limited to laboratories dealing with large numbers of specimens, and reference laboratories where quality control of sera may not be so difficult.

SUMMARY

A method of grouping β-haemolytic streptococci serologically by enzyme-linked immunosorbent assay is described. A comparison of this method with double diffusion in agar gel showed complete correlation of results when highly absorbed grouping sera were used.

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Immunochemical Studies on the Cell-wall Antigen of Group B Streptococcus, Type Ib

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A polysaccharide antigen of group B streptococcus type Ib was extracted from sodium dodecyl sulphate-treated cell walls by trichloroacetic acid. In crossed immunoelectrophoresis the polymer reacted with specific antisera to serotypes Ia, Ib and Ic of group B streptococci and with commercial grouping antiserum to give one precipitin line. However, the antigen did not react with antisera to types II and III. The evidence suggests that this polymer confers the group specificity to type Ib of group B streptococci. The polysaccharide, after purification on DEAE-cellulose, was shown to consist of rhamnose, glucosamine, galactose, glucose, sialic acid and a trace of phosphorus.

INTRODUCTION

The recognition that group B streptococci (GBS) play an important role in the pathogenesis of neonatal infections has generated much interest in these organisms. Their definitive identification is based on serological reaction of the group-specific carbohydrate antigen which is extracted from the cell wall by hot hydrochloric acid (Lancefield, 1933). Early reports indicated that the components of this antigen are polysaccharides of low molecular size and consist mainly of rhamnose (Slade & Slamp, 1962; Curtis & Krause, 1964*a*; Wittner & Hayashi, 1965). Immunochemical studies by sugar inhibition tests of the precipitin reaction between the GBS antigen and specific antisera showed that rhamnose is the principal immunodeterminant portion of the antigen; this suggests that it occupies a terminal position in the polysaccharide (Curtis & Krause, 1964*b*).

More recent studies have concentrated on the immunochemical characterization of the type-specific, or capsular antigens of GBS, since these surface components are theoretically most likely to be recognized immunologically by the infected host (Freimer, 1967; Wilkinson, 1975; Kasper *et al.*, 1979). Capsular antigens extracted by the HCl technique (Lancefield, 1934) have been shown to be partially degraded and immunologically incomplete (Baker *et al.*, 1976). Isolation of the complete native antigen by more subtle extraction procedures allows an acid-labile terminal sialic acid determinant to be identified. Tai *et al.* (1979) were able to isolate from whole cells the Ib type-specific antigen which contained galactose, glucose, *N*-acetylglucosamine and sialic acid in the approximate molar ratio 2 : 1 : 1 : 1. The fact that this antigen cross-reacted with both type Ia and type Ic antisera is attributed to the common determinant Iabc (Lancefield *et al.*, 1975). Absorption studies suggest that the Ib specific determinant and the Iabc determinant are on the same molecule but that sialic acid is not the cross-reactive determinant (Tai *et al.*, 1979).

Various findings thus have shown that the character of GBS antigens is complex. The cell surfaces contain a range of poorly characterized carbohydrate and protein antigens which

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have been extracted by a variety of techniques. In this study we have concentrated solely on the carbohydrate antigen that is covalently linked to the cell wall of type Ib GBS. Its chemical composition and immunological profile have been examined.

METHODS

Strains of group B streptococci. The following strains, representative of the various serotypes, were obtained from the Streptococcal Reference Laboratory, Colindale, London: 0903 (type Ia), H36B (Ib), NCTC 11078 (Ic), NCTC 11079 (II), NCTC 11080 (III).

Antisera. Specific antisera for types Ia, Ib, II and III were raised in New Zealand White rabbits by intravenous injection of formalin-killed, trypsin-treated whole cell preparations. Heat-killed whole cells of strain NCTC 11078 were prepared to obtain antisera specific for type Ic bacteria. The immunization schedule has been described previously (Cumming *et al.*, 1980). Group B antiserum was purchased from Wellcome Reagents. Antisera were not absorbed and their specificity was checked by capillary precipitin tests (Swift *et al.*, 1943) and double diffusion in agarose gel against Lancefield extracts.

Preparation of cell walls and extraction of carbohydrate. Saline-washed bacteria from overnight cultures (6 l) in Todd-Hewitt broth were suspended in ice-cold water and disrupted by passing through a French press (Aminco Inc., Md., U.S.A.) at 48 MPa (7000 lbf in^{-2}). Cell walls and unbroken bacteria were collected by centrifugation at 52000 ***g*** for 15 min at 4 °C and then carefully separated. The suspension of cell walls was heated at 80 °C for 3 min to destroy autolytic enzymes, then washed in water at 4 °C. Protein and membrane components were removed from the suspension by adding an equal volume of 5% (w/v) sodium dodecyl sulphate (SDS) and stirring for 4 h at room temperature (Poxton *et al.*, 1978). To remove SDS, the cell walls were washed by centrifugation at 50000 ***g*** for 10 min in six successive changes of water at 20 °C. Carbohydrate was extracted from 75 mg freeze-dried walls with 10 ml 10% (w/v) trichloroacetic acid (TCA) for 48 h at 4 °C. Walls were collected by centrifugation at 50000 ***g*** for 10 min and TCA was removed from the supernatant fluid by six successive extractions with 10 ml volumes of diethyl ether. Water was removed by rotary evaporation and the extracted carbohydrate was dissolved in 1 ml water.

Purification of carbohydrates was performed by ion-exchange chromatography. Samples of carbohydrate in 5 ml water were applied to a column of DEAE-cellulose previously treated with 1 M-pyridinium acetate (pH 5·3) and eluted by a linear gradient of 100 ml pyridinium acetate (pH 5·3) from 0 to 1 M concentration. Fifty fractions were collected and samples were analysed for carbohydrate.

Lancefield extracts from whole cells. The group antigen extraction was performed by a modified method of Lancefield and Freimer, described previously by Cumming *et al.* (1980).

Analytical techniques. Carbohydrate concentration was estimated by the method of Dubois *et al.* (1956), phosphate analysis was done by the method of Chen *et al.* (1956), and sialic acid was determined by the thiobarbituric acid method of Aminoff (1961).

Crossed immunoelectrophoresis (CIE) techniques. Electrophoresis was carried out with a Shandon Southern apparatus (Camberley, Surrey, U.K.). The technique used was similar to that described by Weeke (1973*b*). Antigen samples (10 µl, containing 1 mg carbohydrate ml^{-1}) were applied to gels of 1% (w/v) agarose (BDH electrophoresis grade). A barbital/glycine/Tris buffer, pH 8·8, described by Weeke (1973*a*) was used throughout. Electrophoresis was at 12·5 V cm^{-1} for 2 h in the first dimension and at 12 V cm^{-1} for 16 h in the second dimension, both at 4 °C. After washing, pressing and drying of the gels, precipitin lines were detected by Coomassie blue stain (Weeke, 1973*a*).

Acid hydrolysis. Samples eluted from the DEAE-cellulose column were hydrolysed in either 2 M-HCl for 4 h at 100 °C or 4 M-HCl for 18 h at 100 °C. Acid was removed by desiccation over NaOH and P_2O_5 in vacuo and the samples were dissolved in 0·5 ml water.

Paper chromatography. Descending paper chromatography was performed on Whatman no. 1 paper with a pyridine/acetic acid/water (6 : 4 : 3, by vol.) solvent. Aminosugars were detected by ninhydrin and reducing sugars by the alkaline silver nitrate reagents (Trevelyan *et al.*, 1950).

Preparation of alditol acetates and gas–liquid chromatography (g.l.c.). Hydrolysed samples were converted to alditol acetates as follows. Hydrolysates (1 ml) were reduced by 10 mg potassium borohydride for 1 h at 20 °C. Excess borohydride was destroyed by addition of glacial acetic acid. Methyl borate was removed from the samples by three successive distillations with methanol. The residue of each sample was then dissolved in 0·5 ml pyridine plus 0·5 ml acetic anhydride and heated for 1 h at 100 °C. Pyridine and excess acetic anhydride were removed by rotary evaporation with successive volumes of toluene until the acetate residues were dry. To remove impurities in the residues, 2 ml chloroform was added followed by vigorous mixing with water using a vortex mixer. The chloroform phase was subjected to rotary evaporation and the resulting samples were redissolved in 0·5 ml chloroform and analysed by g.l.c.

Alditol acetates were identified in a Pye Unicam series 104 chromatograph. A column (2·1 m × 4 mm) containing 3% OV-225 on 100–120 mesh Gas Chrom Q was used for identification of amino sugars; hexoses were identified on a column (1·5 m × 2 mm) of 3% SP-2330 on 100–120 mesh Supelcoport. Gas flow rates for the OV-225 column were 35 ml N_2 min^{-1}, 35 ml H_2 min^{-1} and 525 ml air min^{-1}. Flow rates for SP-2330 were 20 ml N_2 min^{-1}, 20 ml H_2 min^{-1} and 525 ml air min^{-1}. The temperature of both columns was 180 °C rising by 2 °C min^{-1} to 240 °C.

RESULTS

Cell wall and carbohydrate preparations

The yield of SDS-purified cell walls obtained from a 6 l culture volume of type Ib cells was 600 mg. Total carbohydrate extracted from the cell walls was approximately 6 mg. The amount of reducing sugar in the sample was estimated as 3·0 mg by the phenol/sulphuric acid assay.

CIE of TCA-prepared wall antigens

Preliminary studies indicated that the optimum antigen concentration for electrophoresis was 10 µl carbohydrate adjusted to 1 mg ml^{-1} estimated by the phenol/sulphuric acid assay (Dubois *et al.*, 1956). The optimum antiserum concentration to obtain clear precipitin lines in the second dimension of CIE was 0·5 ml antiserum in 3 ml agarose.

Electrophoresis of the Ib antigen extracted from cell walls was performed with sera raised against the five main serotypes of GBS and with commercial grouping antiserum. Single precipitin lines were observed with Ia, Ib (Fig. 1) and Ic antisera and also with the Wellcome grouping antiserum. No reaction was observed with type II or III typing antisera.

To check the specificity of the antisera, 'Lancefield' group antigens were prepared from whole cells of the five serotypes of GBS and examined by the Ouchterlony technique. These group antigen extracts resulted in one line of identity being produced between antigens from all serotypes and the Wellcome grouping antiserum. However, when they were reacted against the typing sera, Ia, Ib and Ic extracts reacted with each other's typing antiserum but not with antisera against types II and III, while group extracts from II and III reacted only with their homologous typing antiserum. This showed that the typing antisera were devoid of group antibodies.

Sialic acid determinations

Purified Ib cell walls were treated with 0·02 M-HCl at 80 °C for periods up to 24 h to extract sialic acid. From a sample (10 mg) of freeze-dried walls 120 µg sialic acid was liberated after 4 h, whereas after 24 h only 81 µg sialic acid was detected. After TCA treatment of walls, the sialic acid content of the extract was 63 µg mg^{-1}; of this, 30·5 µg was in the bound form. Control samples of sialic acid subjected to the same conditions as the test samples showed that, after 4 h heating in HCl, there was a 30% loss of sialic acid, and after 24 h, a 70% loss. The results obtained from cell walls and TCA extract were corrected accordingly for destruction of sialic acid.

Purification and analysis of TCA extract

Extracted carbohydrate preparations from cell walls were subjected to DEAE-cellulose chromatography and eluted with increasing molarities of pyridinium acetate (pH 5·3). Analysis of each sample from the column detected one carbohydrate fraction only, eluting between 0·05 M- and 0·2 M-pyridinium acetate. Crossed immunoelectrophoresis of this fraction showed it to be immunologically similar to the unpurified extract.

Paper chromatography of the purified sample hydrolysed in 2 M-HCl for 4 h at 100 °C resulted in the detection of glucosamine and rhamnose; after hydrolysis in 4 M-HCl for 18 h at 100 °C only glucosamine was detected. Confirmation of these results was obtained by

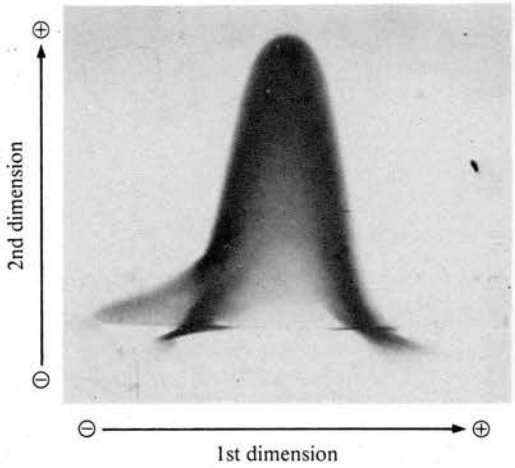

Fig. 1. Crossed immunoelectrophoresis of TCA-extracted GBS type Ib cell-wall antigen (10 µl) against antiserum to GBS type Ib (0·3 ml). This shows a single antigen which is heterogeneous for molecular weight.

Table 1. *Chemical composition of cell wall extract of type Ib GBS, strain H36B*

Component	Molar proportion
Galactose	0·6
Glucosamine	1·0
Glucose	0·2
Rhamnose	1·9
Sialic acid	0·03

analysis of the derivatized hydrolysate by g.l.c. In addition to glucosamine and rhamnose, small amounts of galactose and glucose were identified.

Analysis for phosphate in the sample detected trace amounts only. The complete chemical profile of this antigen, expressed as molar proportions, is summarized in Table 1.

DISCUSSION

In this study an attempt has been made to examine the nature of an antigenic carbohydrate polymer which is covalently bound to the peptidoglycan component of the cell wall of group B streptococci (GBS) of type Ib. Unlike previous reports in which antigens were extracted from untreated whole cells, in this study cell walls were collected and purified by SDS treatment, thereby removing all non-covalently bound polymers. Only one carbohydrate fraction was extracted from the walls after prolonged treatment with TCA; this polymer was shown to have a net negative charge by its binding capacity to DEAE-cellulose. Chemical analysis of the extract resulted in the identification of rhamnose, glucosamine, galactose, glucose, sialic acid and a trace of phosphorus. The relative proportions of these compounds are essentially similar to the composition of the group-specific antigen isolated from whole cells of GBS type Ia by Kane & Karakawa (1978). The presence of trace amounts of phosphorus and the TCA lability of the bond between the isolated carbohydrate and peptidoglycan suggest that a linkage unit containing phosphodiester bonds might be present. This would be analogous to the peptidoglycan–teichoic acid linkage in other Gram-positive bacteria (Coley *et al.*, 1978).

Specific antigen–antibody reactions were demonstrated by CIE. Single precipitin lines were detected when the Ib antigen was reacted with antisera raised against serotypes Ia, Ib, Ic and with the commercial grouping antisera. No reaction between the extracted antigen and typing antisera for types II and III could be detected.

These findings indicate that type I strains of GBS possess a carbohydrate antigen which is covalently linked to the cell wall peptidoglycan in type Ib streptococci. This antigen does not confer sub-type specificity (Ia, Ib, Ic), and antibodies to this antigen do not appear to be provoked by whole cell antigen preparations of types II or III. It is surprising that the group-specific antigen of type I strains (i.e. the carbohydrate cell wall antigen) does not react with typing antisera to types II or III, although grouping antigens of types II and III react with commercially available group B serum. The type-specific antigens present in the 'Lancefield' extract are not covalently bound to the cell wall, as they can be removed by the SDS procedure. These are probably capsular or membrane-bound in their location within the B complex.

During preparation of this manuscript, Wagner *et al.* (1980) demonstrated by immunoelectron microscopy that the group-specific polysaccharide antigen of GBS traverses the whole of the cell wall. This is in complete agreement with the findings in this study, which indicate that the group-specific polysaccharide is the secondary wall polymer covalently bound to peptidoglycan.

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Further studies on the immunochemical nature of the cell-wall antigens of group - B streptococcus, type Ia

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Definitive identification of group-B streptococci (GBS) is based on serological reaction of the 'group-specific' carbohydrate antigen which can be extracted from the cell wall by hot HCl[10]. Early reports indicated that the components of this antigen are polysaccharides of low-molecular size and consist mainly of rhamnose[7-12]. Sugar inhibition studies of the GBS antigen-antiserum reaction suggested that rhamnose forms the principal immunodeterminant of the antigen and occupies a terminal position in the polymer. In addition, the use of more subtle extraction procedures made possible the identification of acid-labile sialic acid in the cell-wall antigen[9].

Recently, immunoelectron microscopy studies[11] have demonstrated that the 'group-specific' antigen of GBS traverses the whole of the cell wall, whereas the 'type-specific' antigen is found only on the outer surface of the wall.

The aims of the present study were:

1) to extract and purify the carbohydrate antigen which is covalently bound to peptidoglycan of GBS, type Ia cell walls,
2) to examine the serological character of the antigen; and
3) to perform a chemical analysis of the antigen.

Materials and methods

Bacteria. GBS, type Ia 09OR, were obtained from the Streptococcus Reference Laboratory, Colindale, London.

Antisera. Specific antisera for GBS types Ia, Ib, Ic, II and III were raised in New Zealand white rabbits by a method described previously[5].

Preparation of cell walls. Saline-washed bacteria from a 10 litre overnight culture in Todd-Hewitt broth were disrupted by passage through a French pressure cell (Amino Inc., Maryland, USA) at 7000 p.s.i. Cell walls were collected by centrifugation at 50,000 *g*. The suspension of walls was heated at 80°C for 3 min to destroy autolytic enzymes, and washed in three successive volumes of water. Protein and membrane components were removed from the suspension by adding an equal volume of boiling 5% (w/v) sodium dodecyl sulphate (SDS) and stirring for 4 h at 20°C. SDS was removed by washing the walls in six changes of water, and the purified walls were freeze-dried.

Extraction of antigen. Cell walls were divided into three portions and carbohydrate extraction was performed by the following methods.

Walls (100 mg in 10 ml water) were suspended in 10 ml of 10% (w/v) trichloroacetic acid (TCA) and stirred for 8 h at 4°C. The supernate containing carbohydrate as collected by certrifugation at 50,000 *g* for 10 min. TCA was removed by six successive extractions with 10 ml volumes of diethyl either. Concentration os carbohydrate was adjusted to 1 mg/ml in water.

2. Walls (100 mg) were subjected to a similar procedure as above, except that extraction was performed with TCA for 48 h at 4°C.

3. Carbohydrate was extracted from the third portion of walls with 0.5 M NaOH for 2 h at 20°C by a method described by Anderson & Archibald[2]. Sodium ions were removed by ion exchange chromatography on a column of Dowex 50 NH_4^+ form).

Purification of antigens. Free sialic acid was removed from the TCA (48 h) extracted by fractioning on a column of Sepharose G25 (Pharmacia). This antigen was further purified with DEAE-cellulose. Material was eluted from the column with a linear concentration gradient (0→1 M) of 100 ml of pyridinium acetate buffer, pH5.3.

The NaOH extract was purified by DEAE-cellulose chromatography.

Serological techniques. Serological profiles of the unpurified and purified extracts were performed against 'type-specific' GBS antisera using crossed immunoelectrophoresis, described previously (Cumming, Ross & Poxton, 1981).

Analytical techniques. Carbohydrate concentration was estimated by the method of Dubois et al[8], phosphate analysis by the method of Chen, Toribara & Warner[3], and sialic acid was determined by the thiobarbituric acid method[1].

Sugars were identified by paper chromatography and gas-liquid chromatography. Conditions and methods of alditol acetate preparation were described prreviously[6].

Results

CIE of wall extracts. Serological reaction of the unpurified NaOH cell wall extract with 'type-specific' Ia, Ib and Ic GBS antisera resulted in the formation of single precipitin lines (line i). No reaction was detected between the NaOH extract and antisera to types II and III GBS.

Two precipitin lines (i and ii) were observed when the unpurified TCA (8 h) cell wall extract was reacted with Ia 'type-specific' antiserum. Against types Ib and Ic antisera single precipitin lines (line i) were produced. There was no reaction with antisera types II and III. CIE of the unpurified TCA (48 h) cell wall extract with Ia antiserum resulted in two precipitin lines (i and ii), although this extract consisted predominantly of antigen forming line ii. No reaction was detected with Ib antiserum, whereas there was a faint reaction with Ic 'type-

Table Chemical composition of NaOH and TCA cell wall extracts (strain 090R)

Extract	Content (molar proportion of)				
	Galactose	Glucose	Glucosamine	Rhamnose	Sialic acid
NaOH (antigen (i)	0.6	0.2	1	2	0.06
TCA (48h) (antigen ii)	2	1	1	2.6	trace

specific' antiserum (line i). Again, no reaction occurred with antisera of GBS serotypes II and III.

Purification of wall extracts. Following removal of free sialic acid from the TCA (48 h) extract by fractionation on Sepharose G25, this extract and the NaOH extract were further purified by DEAE-cellulose chromatography. Analysis of the column fractions indicated that one major carbohydrate polymer in each was extracted. Serological testing by CIE showed that the purified NaOH extract was identical with the unpurified extract, i.e. gave rise to line i. The purified TCA (48 h) extract contained only antigen, which reacted with acetate preparation were described previously[6], homologous antiserum to form precipitin line ii.

Chemical analysis. Constituents of the NaOH and TCA (48 h) extracts are expressed as molar proportions in the table. In addition, traces of phosphorus were found in both extracts.

Discussion

The NaOH extraction technique made possible the isolation of a single carbohydrate antigen from GBS, type Ia cell walls. This sialic acid-containing polymer is similar in composition to the 'group-specific' antigen isolated from GBS type Ib cell walls[6]. Treatment of cell walls by cold TCA for 8 h caused the release of an antigenic complex, predominantly similar to the NaOH extract, but containing a partially degraded polymer, lacking sialic acid, and exhibiting a different electrophoretic mobility. An increase in the duration of TCA treatment to 48 h resulted in further destruction of sialic acid, producing an antigenic complex which is serologically distinct from the NaOH-extracted antigen. Chemical analysis of the antigens indicated that the main difference between the two is the absence of sialic acid in the TCA (48 h) extract.

The presence of trace amounts of phosphorus and the TCA-lability of the bond between the isolated carbohydrates and peptidoglycon suggest that a phosphodiester bond analogous to the peptidoglycan-teichoic acid linkage described in other Gram-positive bacteria[4] may be present in GBS, type Ia.

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THE ISOLATION AND IMMUNOCHEMICAL CHARACTERISATION OF A CELL-WALL CARBOHYDRATE AND A MEMBRANE LIPOCARBOHYDRATE ANTIGEN OF GROUP B STREPTOCOCCUS, TYPE II

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SUMMARY. Two distinct carbohydrate antigens were isolated from the cell surface of group B streptococcus, type II. One antigen was extracted from SDS-purified cell walls by cold trichloroacetic acid and contained galactose, glucose, rhamnose, glucosamine and sialic acid in the approximate molar proportions 1·7:1·0:3·4:0·9:0·21 respectively. The serological activity of this polymer indicated that it is the group-specific antigen common to all group B streptococci. The second antigen was extracted by phenol from cell membranes and contained galactose, glucose, glucosamine, phosphorus and fatty acid in a molar ratio of 1·6:1·0:0·35:2·6:0·016 respectively. This antigen was shown to be specific for type II, group B streptococcus.

INTRODUCTION

Lancefield (1933) demonstrated that beta-haemolytic streptococci could be differentiated into several serological groups, labelled A–E, by the presence of a group-specific carbohydrate antigen or C-substance. Further studies by Lancefield (1934, 1938) divided group B streptococci (GBS) into four serological types by the use of acid extracts of type-specific surface or capsular polysaccharide antigens. These types were designated Ia, Ib, II and III, and later type Ic was identified by the detection of the type Ibc protein antigen on the surface of strains possessing a Ia polysaccharide antigen (Wilkinson and Eagon, 1971).

Early reports on the immunochemical character of GBS antigens indicated that the group-specific antigen is composed mainly of rhamnose (Slade and Slamp, 1962; Wittner and Hayashi, 1965). In addition, rhamnose was shown to be the principal immunodeterminant in the antigen, suggesting that it occupied a terminal position in the polysaccharide (Curtis and Krause, 1964). Recently, the chemical profile of the group-specific antigen from GBS Ib was determined (Cumming, Ross and Poxton, 1981).

In laboratory animals, GBS have been shown to elicit the production of multiple populations of protective antibodies, particularly against the surface type-specific antigens (Lancefield, McCarty and Everly, 1975). When extracted with hot HCl, these

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antigens are partially degraded and immunologically incomplete (Baker, Kasper and David, 1976). Extraction of GBS type II polysaccharide antigen with cold trichloroacetic acid (TCA) gave a polymer containing two serological determinants, whereas a partial antigen with only one of the determinants was obtained by extraction with boiling HCl (Freimer, 1967). D-galactose was identified as the determinant of the HCl-extracted polysaccharide. The second determinant, present only in the TCA extract was not identified. Recent studies have identified sialic acid as the important second immunodeterminant (Kane and Karakawa, 1978; Tai, Gotschlich and Lancefield, 1979; Kasper *et al.*, 1979).

The numerous reports on the immunochemical nature of the cell surfaces of GBS have indicated a complexity of antigenic structure only partially understood. The present study investigated two distinct classes of antigen extracted from GBS type II. The purified cell-wall carbohydrate antigen was examined serologically and chemically, and the first investigation of the cell-membrane polymer (teichoic acid or analogue) was undertaken.

Materials and methods

Strains of group B streptococci. The following strains, representing the various serotypes, were obtained from the Streptococcus Reference Unit, Division of Hospital Infection, Central Public Health Laboratory, Colindale, London: 090R (type Ia), H36B (Ib), NCTC11078 (Ic), NCTC11079 (II), NCTC11080 (III).

Antisera. Specific antisera for GBS types Ia, Ib, II and III were raised in New Zealand white rabbits by intravenous injection of formalin-killed, trypsin-treated whole cells. Heat-killed (56°C for 30 min) whole cells of strain NCTC11078 were prepared to obtain antisera for type Ic GBS. The immunisation schedule has been described previously (Cumming *et al.*, 1980). Group B antiserum was purchased from Wellcome Reagents, Beckenham, Kent. Antisera were not absorbed and their specificity was checked against homologous and heterologous Lancefield extracts of GBS by double diffusion in agar gel.

Preparation of type II cell-wall extracts. Saline-washed bacteria from overnight cultures (10L) in Todd-Hewitt broth were suspended in ice-cold water and disrupted by passing through a French press (Aminco Inc., Silver Spring, MD., USA) at 48MPa (7000 lbf in^{-2}). The suspension from the French press was centrifuged at 50 000 *g* for 15 min at 4°C. The supernate containing membranes was removed, freeze-dried and retained for subsequent extraction of antigen, while the upper layer of cell walls was carefully separated from the small pellet of unbroken cells. The suspension of cell walls in water was heated at 80°C for 3 min to destroy autolytic enzymes, then washed in water at 4°C. Non-covalently-linked protein and membranes were removed from the suspension by adding an equal volume of 5% (w/v) sodium dodecyl sulphate (SDS) and stirring for 4 h at room temperature. To remove SDS, the cell walls were washed by centrifugation at 50 000 *g* for 10 min in six successive changes of water at 20°C. Carbohydrate was extracted from the walls with 10% (w/v) trichloroacetic acid, as described previously by Cumming *et al.* (1981). Purification of carbohydrate was performed by ion-exchange chromatography. Samples in 2 ml of water were applied to a column (30 cm × 1·5 cm) of DEAE cellulose previously treated with 1M-pyridinium acetate (*p*H 5·3) then equilibrated with water and eluted with 50 ml of water and 50 ml of 1M pyridinium acetate (*p*H 5·3) to give a linear gradient from 0–1M concentration. Fifty fractions of 2 ml were collected and samples were analysed for carbohydrate.

Preparation of type II cell-membrane extracts. After French pressing, unbroken bacteria and cell walls were removed by centrifugation and the supernatant fluid containing cell membranes was freeze dried and weighed. Membranes (1 g) were de-fatted by stirring for 18 h at room temperature in 200 ml of chloroform-methanol (2:1 v/v). The membranes were filtered, rinsed with 200 ml chloroform-methanol mixture, and dried in air. Lipocarbohydrates were extracted from the membranes with cold 80% aqueous phenol as described by Coley, Duckworth

and Baddiley (1975). Nucleic acids were removed by incubation of the extract with RNase A and DNase I (Sigma Chemical Company, London) at concentrations of 10 μg/ml in 0·2 M sodium acetate-acetic acid buffer (*p*H 5·0) containing 10^{-3} M $MgCl_2$, under toluene for 48 h at 37°C. To ensure complete removal of contaminants, the aqueous phenol extraction procedure was repeated. Further purification of the extract was performed on a Sepharose 6B (Pharmacia Fine Chemicals, Sweden) column (60 cm × 1·6 cm). Fractions (3 ml) were eluted by an upward flow of 0·2 M ammonium acetate buffer, *p*H 6·9, containing sodium azide 0·01%. Fractions were analysed for carbohydrate and assessed for antigen content with GBS antisera by fused rocket immunoelectrophoresis.

Analytical techniques. Carbohydrate was estimated by the method of Dubois *et al.* (1956), phosphate analysis was by the method of Chen, Toribara and Warner (1956), and sialic acid was determined by the thiobarbituric acid method of Aminoff (1961).

Immunoelectrophoresis techniques. Electrophoresis was carried out with a Shandon Southern apparatus (Camberley, Surrey, UK). Fused rocket immunoelectrophoresis (Svendsen, 1973) against GBS type II antiserum in the gel was used to assess the antigen content of cell-wall and membrane fractions from the chromatography columns. Pooled antigen-containing fractions were subjected to crossed immunoelectrophoresis (CIE) techniques, described previously by Cumming *et al.* (1981). Simple immunodiffusion experiments in agar gel were performed by the method of Ouchterlony (1948).

Acid hydrolysis. Purified cell-wall and membrane samples were hydrolysed in 2 M HCl for 4 h at 100°C. Acid was removed by desiccation over NaOH and P_2O_5 in a vacuum and the samples were dissolved in 0·5 ml water.

Paper chromatography. Descending paper chromatography was performed on Whatman no. 1 paper with a butan-1-ol/pyridine/water (6:4:3, by volume) solvent. Aminosugars were detected by ninhydrin and reducing sugars by the alkaline silver nitrate reagents (Trevelyan, Procter and Harrison, 1950).

Preparation of methyl esters of fatty acids. Methyl esters of the fatty acids of the lipid portion of the cell-membrane extract were prepared by heating in 0·5M HCl in methanol at 65°C for 2 h. The methyl esters were partitioned into ether and the constituents were analysed by gas-liquid chromatography (GLC) on columns of SP2330 3% on Chromosorb W.

Preparation of alditol acetates. Hydrolysed carbohydrates were converted to alditol acetates using methods described by Cumming *et al.* (1981).

Results

Cell-wall and carbohydrate preparations

The yield of SDS-treated GBS type II cell walls from 10L of culture was 950 mg. Total carbohydrate extracted with trichloroacetic acid was approximately 10 mg.

Purification and analyses of cell-wall extract

Fractions eluted from the DEAE-cellulose column were collected and assayed for carbohydrate. One major peak (W1) was eluted between 0·2M and 0·26M pyridinium acetate buffer (fig. 1). Two additional peaks (W2 and W3) were detected; they were eluted between 0·38M–0·58M and 0·62M–0·82M pyridinium acetate buffer, respectively.

Chemical analyses were performed on material from peaks W1 and W2 only. Examination of acid hydrolysates of W1 by descending paper chromatography showed the presence of galactose, glucose, glucosamine and rhamnose. These constituents were confirmed by GLC of alditol acetate derivatives from which molar proportions were calculated. In addition, significant amounts of sialic acid and trace

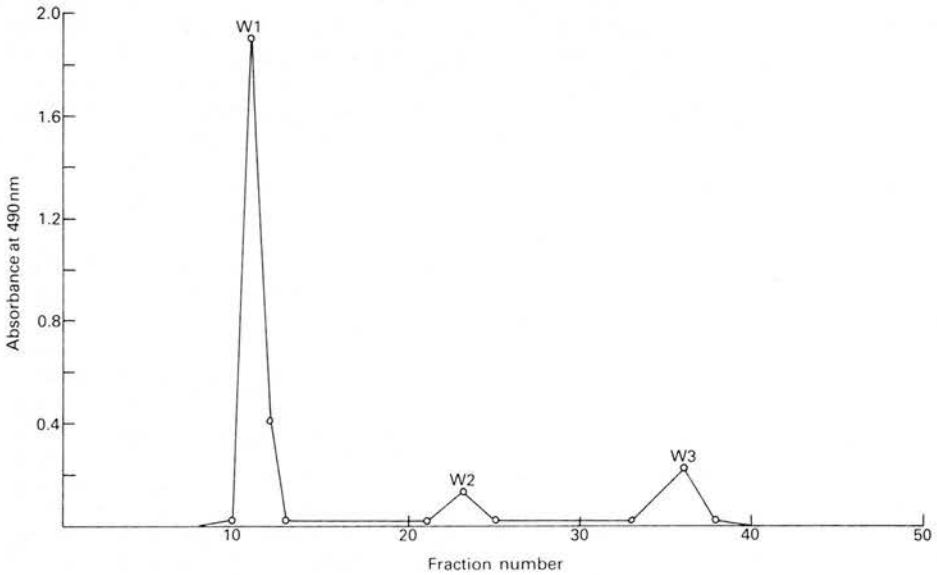

FIG. 1.—Purification of a TCA extract of SDS-treated cell walls of GBS, type II by ion exchange chromatography on a DEAE-cellulose column. Fractions (2 ml) were eluted with a linear gradient (0 to 1 M) of pyridinium acetate, pH 5·3. Carbohydrate was assayed by the method of Dubois *et al.* (1956).

amounts of phosphorus were identified in the fraction. The complete chemical composition of the polymer W1, expressed as molar proportions, is shown in table 1.

Analyses of W2 showed the presence of ribose and trace amounts of those constituents found in extract W1, except sialic acid which was not detected.

CIE of cell-wall fractions

The antigenic nature of the cell-wall fractions was assessed with 'type' and 'group-specific' GBS antisera by CIE. Material from peak W1 produced a single precipitin line in gel with type II and commercial GBS grouping antisera. No reaction occurred with type Ia, Ib, Ic or III 'type-specific' antisera. Similar results were obtained with peak W2 and the GBS antisera. There was however, a slight difference

TABLE I

Chemical composition of material from peak W1 from DEAE chromatography of a TCA extract of SDS-treated cell walls of GBS, type II

Component	Molar proportions
Galactose	1·7
Glucose	1·0
Rhamnose	3·4
Glucosamine	0·9
Sialic acid	0·21
Phosphorus	trace

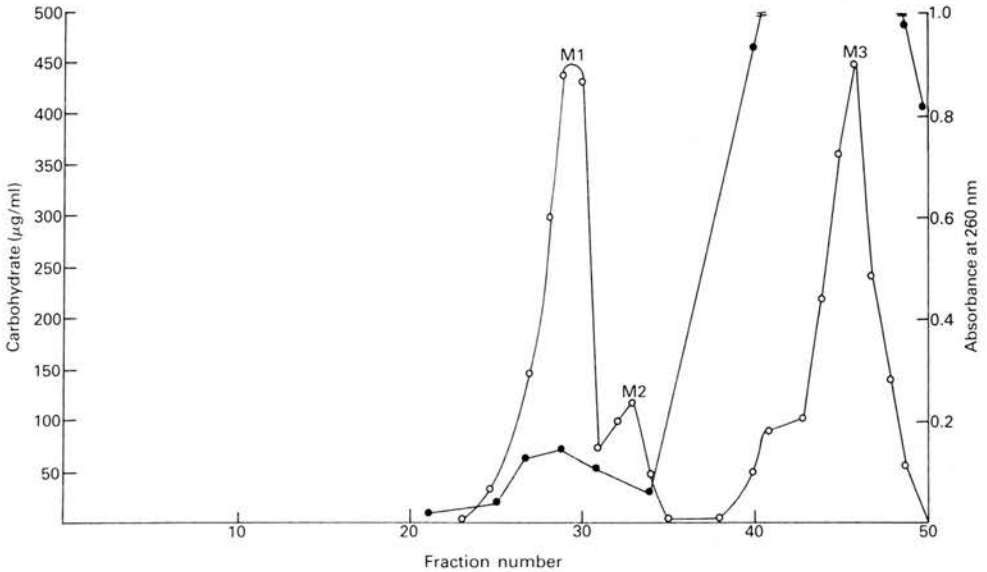

FIG. 2.—Purification of a phenol extract of the cell membranes of GBS, type II on a Sepharose 6B column. Fractions (3 ml) were collected and assayed for nucleic acid at 260 nm (●) and carbohydrate (○).

in electrophoretic mobility in gel between the two fractions when they were run in tandem gels against commercial grouping antiserum.

No reaction was detected between material from peak W3 and the GBS antisera.

Cell-membrane preparation

The yield of freeze-dried cell membranes from an overnight culture of GBS type II in 10L of Todd-Hewitt broth was 1·26 g. Following phenol-extraction procedures, 47·5 mg of extract was collected.

Analyses of cell-membrane extract

Material extracted by phenol from type II cell membranes was applied to a Sepharose 6B column and the fractions assayed for carbohydrate, phosphorus and nucleic acid. The elution profile is shown in fig. 2. Three distinct carbohydrate peaks

TABLE II

Carbohydrate composition of material from peak M1 from Sepharose 6B chromatography of a cold aqueous phenol extract of cell membranes of GBS type II

Component	Molar proportion
Galactose	1·6
Glucose	1·0
Glucosamine	0·35
Phosphorus	2·6

TABLE III

Lipid composition of material from peak M1 from Sepharose 6B chromatography of a cold aqueous phenol extract of cell membranes of GBS type II

Component	Percentage of total lipid
Palmitic acid (C:16)	74
Stearic acid (C:18)	17·5
Oleic acid (C:18.1)	8·5

(M1, M2 and M3) were detected. Analysis of peak M1 for carbohydrate and lipid was performed (tables 2 and 3). Sialic acid was not detected. Chemical analyses of material from peaks M2 and M3 were not performed.

CIE of cell-membrane fractions

Material from peak M1 reacted only with antiserum against type II GBS to produce one characteristic precipitin line in agar gel, but material from M2 reacted with type II antiserum and with commercial grouping antiserum forming single precipitin lines. Simple immunodiffusion experiments indicated that with both type II and grouping antisera, material from peak M2 formed precipitin lines of complete identity with material from cell-wall peak W1. Lines of identity were not detected with either of these extracts or material from peak M1.

DISCUSSION

The results described in this study indicate that two distinct antigenic carbohydrate polymers can be isolated from cell walls and membranes of GBS, type II. The chemical composition of the main antigenic peak (W1) from the TCA extract of SDS treated cell walls is qualitatively similar to the group-specific antigen isolated from GBS type Ib (Cumming *et al.*, 1981). The second antigenic cell-wall polymer (W2) differed chemically from the material in peak W1 only in its lack of sialic acid. The absence of sialic acid in this polymer almost certainly accounted for the difference in electrophoretic mobility between the two antigens. In simple immunodiffusion experiments, however, the two antigens produced lines of partial identity when reacted with commercial group-specific antiserum. This pattern of serological activity is similar to that of the group-specific extracts from GBS, type Ia (Cumming, Ross and Poxton, 1982).

The fact that type-specific antigens were removed from cell walls by SDS treatment indicates that these antigens are not covalently bound to peptidoglycan. This opposes the view of Tai *et al.* (1979) who suggested that the capsular type-specific antigens are covalently bound to the cell wall.

Two distinct antigenic polymers were detected in material extracted from type II membranes by cold phenol. Serological reaction of material from peak M1 showed its specificity for GBS type II only, whereas peak M2 produced the same serological profile as the group-specific cell-wall polymer (W1). Immunodiffusion experiments

with material from the two peaks (W1 and M2) confirmed their serological similarity. This agrees with the observation of Coley *et al.* (1975) that wall teichoic acids of certain gram-positive bacteria are often found in the second peak eluted from a Sepharose 6B column.

Chemical analysis of the type-specific antigen (M1) showed it to be a phosphorus containing lipocarbohydrate. This polymer should not be classified as a teichoic acid, however, since it does not contain ribitol or glycerol phosphate. The composition of the carbohydrate portion of the molecule is similar to the type-specific antigen of GBS type II described by Freimer (1967), except for the absence of sialic acid in the membrane polymer. This result implies that the GBS type-specific antigens, commonly referred to as capsular, surface, or cell wall associated antigens, are indeed membrane bound structures.

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THE ISOLATION OF A LECTIN-LIKE MOLECULE FROM CORYNEBACTERIUM PARVUM (NCTC 10390)

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SUMMARY A mannose specific lectin has been isolated by affinity chromatography from the cell wall of *C. parvum*. Polyacrylamide gel electrophoresis indicates that the lectin molecules lie in the molecular weight range 57,000–72,000. It appears that *C. parvum* like *E. coli* and salmonellae express lectins that bind to cells expressing mannose in their membranes. This may partly account for the interaction between *C. parvum* and the macrophage leading to the various immunological phenomena associated with *C. parvum* administration.

INTRODUCTION

FOR some time it has been known that anaerobic coryneforms including *Corynebacterium parvum* (*C. parvum*) are able to bring about activation of the cells of the mononuclear phagocyte system (1) and the process appears to be causally related to enhanced resistance to tumour growth in animals (2). The characteristics and consequences of the binding of *C. parvum* to mouse peritoneal macrophages have been studied in detail (3, 4). A "lectin-like" receptor specific for bacterial cell wall sugars was found in the macrophage membrane and was responsible for binding *C. parvum*. Binding was also associated with changes in phospholipid metabolism prior to activation. Amongst the bacterial cell wall sugars recognised by the lectin-like receptor is D-mannose. The involvement of this sugar was of interest in view of earlier findings that D-mannose could inhibit the haemagglutinating activity of various strains of enteric bacteria (including *Salmonella*, *Klebsiella*, *Shigella* and *Proteus*) which, in the absence of D-mannose, will produce haemagglutination (5, 6, 7). It therefore seemed necessary to investigate the possibility that *C. parvum* binding to macrophages might in part be due to a lectin-like binding site present in the cell wall of the bacterium itself.

The present paper provides evidence for such a mechanism of attachment, involving a lectin-like molecule on the cell surface of *C. parvum* which recognises mannose residues.

MATERIALS AND METHODS

Growth of Bacteria

C. parvum (10390) obtained from the National Collection of Type Cultures Colindale was used throughout. The bacteria were grown in stationary, anaerobic culture for 48 hr at 37°C in horse digest broth containing 3% (w/v) glucose. The cells were harvested by sedimentation at 12,000 g for 10 min and washed once in 0·5% NaCl at 4°C.

Agglutination Assay

Cells of *Saccharomyces cerevisiae* were prepared as described by Eshdat *et al.* (8). The assay was performed on a microscope slide by mixing 10 µl of the solution under test with 5 µl of phosphate buffered saline (PBS) (pH 7·4) and 10 µl of the yeast cell suspension. Agglutination was monitored by the naked eye.

Preparation of the Lectin-containing Extract

The lectin was extracted using the technique of Eshdat *et al.* (8). The washed cells (41 g) were homogenised in 200 ml of PBS containing 850 mM sodium chloride and 0·03 mM phenylmethanesulphonyl fluoride, in a Sorvall Omnimixer (30 min, 0°C). The cells were sedimented by centrifugation at 10,000 g for 20 min, then resuspended in 100 ml of the homogenisation buffer and rehomogenised and resedimented under the same conditions. The combined supernate (300 ml) was centrifuged twice at 5000 g for 10 min to remove any remaining bacteria. This yielded a pale straw-coloured solution which was centrifuged at 48,000 g for 3 hr to produce a pellet below a straw-coloured supernate. The pellet was suspended in 5 ml of PBS.

Protein Assay

The protein assays were performed according to the method of Lowry *et al.* (9).

Affinity Chromatography of the Crude Extract

Yeast mannan was linked to Sepharose 4B as described by Uy and Wold (10). The prepared Mannan-Sepharose 4B was used to pack a 7 cm × 1½ cm column, which was equilibrated in PBS (pH 7·4) containing 0·2% sodium azide. The crude extract from *C. parvum* (2 ml) was centrifuged lightly to remove any solid material from the solution. The column was loaded with 660 µl of this solution (containing 2 mg of protein) and washed through with 18 ml of PBS. The eluate was collected in 1 ml fractions, each of which was screened for protein. A further eighteen 1 ml fractions were then collected with 0·1 M α-methyl-D-mannoside (αMM), in 0·9% sodium chloride solution as the eluting buffer. These were also analysed for protein. The fractions eluted with αMM were dialysed against PBS overnight, and then against distilled water for a further 5 hr, to remove the αMM. The fractions were concentrated by freeze-drying prior to carrying out the agglutination assay.

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Polyacrylamide Gel Electrophoresis (P.A.G.E.)

P.A.G.E. of selected fractions was performed in the presence of 1% sodium dodecyl sulphate (SDS) on 10% polyacrylamide slab gels using the discontinuous buffer system of Laemmli (11).

Electron Microscopy

A broth culture of *C. parvum* was fixed with 0·25% formaldehyde, and the bacteria harvested at 1000 g for 14 min. The cells were gently washed in distilled water and resedimented. The pellet was resuspended in distilled water to produce a milky suspension, one drop of which was placed on a microscope slide. Two Formvar-coated copper grids were placed onto the suspension and left for 1 min. Excess suspension was removed with a piece of filter paper, and the grids inverted. 2% phosphotungstic acid (PTA) was applied for 20 s and excess PTA removed with filter paper. The grids were dried in a desiccator before examination in a Hitachi HU 12A electron microscope.

Results

The crude extract produced by high speed centrifugation (48,000 g) of the homogenate did not show agglutinating activity with glutaraldehyde-treated yeast cells. Fractionation of the crude extract on the Mannan-Sepharose affinity column as shown in Figure 1 led to several protein peaks with that at fractions 15 and 16 containing strong agglutinating activity for the yeast. In an attempt to elute further agglutinating material αMM was added to the column but this treatment failed to elute a protein peak that contained strong agglutinating activity (fig. 1). This suggested that the lectin was not firmly bound to the column and that the majority of the material capable of agglutinating yeast comes off in the buffer eluate.

Table 1 shows fractions and combined fractions used in P.A.G.E. analysis. They were freeze-dried and redissolved in 100 μl of distilled water (except fraction 27 which was dissolved in 50 μl distilled water), and rechecked for agglutinating activity. The table shows that the main agglutinating activity was retained in fractions 15 and 16.

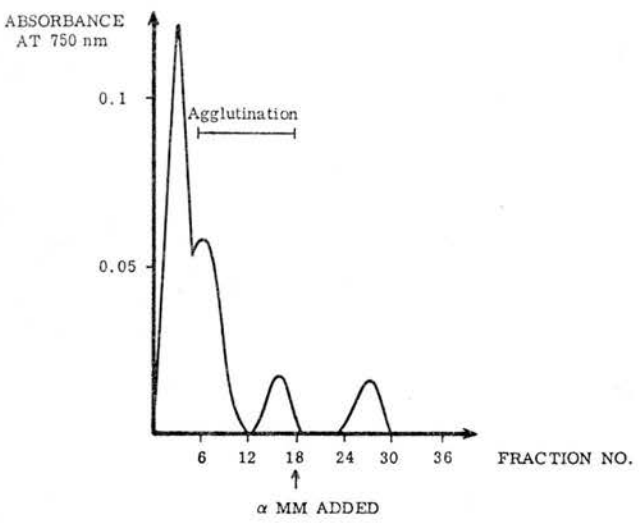

FIG. 1. Protein assay from affinity chromatography of 2 mg of crude extract from *C. parvum* on mannan-linked Sepharose 4B.

Table 1 Data relating to samples run on SDS–P.A.G.E.

Fraction	*Approximate mass of protein*	*Agglutination Assay*
3	230 μg	Negative
4 and 5	240 μg	Negative
6 and 7	210 μg	Positive
15 and 16	100 μg	Very strong
27	50 μg	Very weak

The results of SDS–P.A.G.E. (fig. 2) gave an indication of the protein bands associated with agglutination of the mannan-expressing yeast cells. Repeated gel analysis resulted in the finding that agglutinating activity was confined to two doublet protein bands and possibly a third band in the molecular weight range 57,000–72,000 which were consistently associated with agglutinating activity. Also of interest is the finding that in the fraction eluted with αMM, three further bands of approximate molecular weights, 12,000, 16,000 and 30,000 appear though they are not, apparently, associated with strong agglutination of yeast cells.

Electron microscopy of a preparation of *C. parvum* negatively stained with 2% PTA showed that many of the cells possess structures resembling fimbriae projecting from the cell surface (fig. 3).

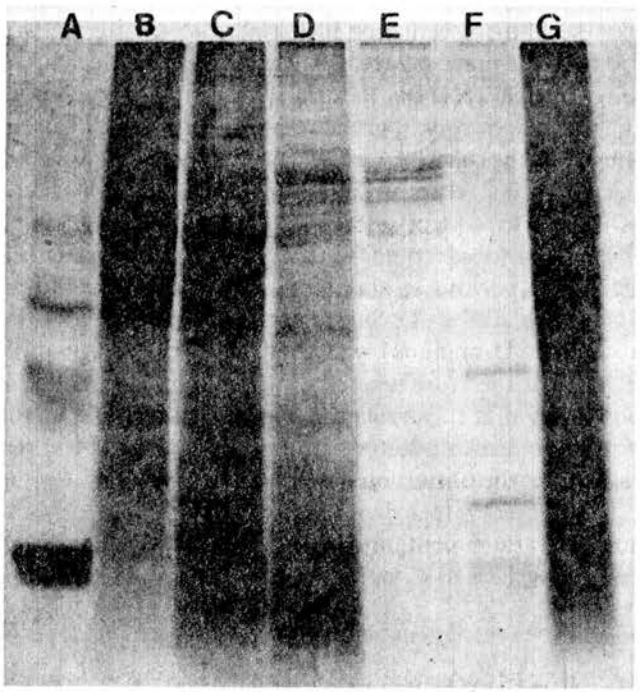

FIG. 2. SDS–P.A.G.E. in a 10% polyacrylamide gel slab of fractions from affinity chromatography of *C. parvum* extract: (a) molecular weight marker; (b) fraction 3; (c) fractions 4 and 5 combined; (d) fractions 6 and 7 combined; (e) fractions 15 and 16 combined; (f) fraction 27; (g) crude extract.

FIG. 3. Electron micrograph of cells of *C. parvum* (10390) negatively stained with 2% P.T.A. × 75,000.

DISCUSSION

Whole *C. parvum* (10390) organisms will agglutinate *Saccharomyces cerevisiae* cells. The results indicate that material containing the agglutinating activity can be extracted from the organism by mild homogenisation and affinity chromatography fractionation. The crude extract itself did not agglutinate yeast cells prior to purification. After fractionation a highly active component was obtained that increased in agglutinating activity along with increasing homogeneity. Thus the fraction shown in band (E) (fig. 2) gave the strongest reaction of the fractions tested. It would therefore appear that the presence of contaminating proteins interferes with the binding reaction between the lectin molecule on the bacterial surface and the mannan residues on the yeast cell.

The results of SDS–P.A.G.E. indicate that the active lectin molecules are proteins in the molecular weight range 57,000–72,000. Invariably lectin activity was shown to be associated with two or three bands, which appear as "doublets" after electrophoresis (fig. 2). A possible explanation for this is that the mannose-specific activity is present as distinct iso-lectins, a finding not uncommon among molecules of this type. A further phenomenon revealed by the SDS–P.A.G.E. is the presence of three lower molecular weight bands in the fractions eluted with αMM. These bands, in the molecular weight positions of 12,000, 16,000 and 30,000, may represent sub-units of the active agglutinin, especially since lectin-like molecules can often exist in several forms, each of differing valency. In order to produce agglutination of the yeast cells, the lectin molecule must obviously be at least divalent. It is interesting that when these bands were present in the absence of the higher molecular weight agglutinin molecules, agglutination was very weak. This might in part be due to the inhibitory effect of contaminating αMM that was not removed even after extensive dialysis.

The hair-like projections from the cell surface, revealed by electron microscopy, may act as probes which can carry the lectin molecules across the high potential energy barrier which exists at a short distance between the bacterial cell and the cell to which it is attaching. Unless this barrier is overcome, there can be no interaction between the bacterial lectin and a mannose residue on, for example, a yeast cell or macrophage membrane, and thus no adhesion can take place (12). The present findings extend our earlier observations that the binding of *C. parvum* to mouse macrophages can be blocked by pretreatment of the bacterium with mannose (13) in the same way that *E. coli* and salmonellae binding to epithelial cells has been shown to be inhibited by mannose (8). It seems likely that this type of binding to cells bearing mannose in their membranes may help to explain the adjuvant and anti-tumour properties of *C. parvum* mediated through macrophages (14).

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BINDING OF TYPE-III GROUP-B STREPTOCOCCI TO BUCCAL EPITHELIAL CELLS

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SUMMARY. A binding assay was used to study the attachment of type-III group-B streptococci (GBS) to buccal epithelial cells. Results indicate that an adhesin, with the characteristics of a protein, is the molecule at the streptococcal cell surface responsible for attachment to the buccal cells. The bacterial adhesin probably recognises a sugar on the surface of the mucosal cell, because periodate oxidation of the buccal cells caused a significant reduction in subsequent adherence of GBS. A sonicate of type-III GBS blocked the binding of the organism to buccal cells. The effects of physical and chemical modifications of the sonicate on its ability to prevent bacterial attachment are described; these corroborate the evidence gained from heat and periodate treatments of the buccal cells and GBS. Results suggest a lectin type of attachment mechanism for type-III GBS which can be blocked by N-acetyl-D-glucosamine, rather than attachment by means of a lipoteichoic acid as described for group-A streptococci.

INTRODUCTION

It is widely accepted that adherence of bacteria to mucosal cells is important in the initiation of many types of microbial disease (Gibbons and van Houte, 1971). Group-B streptococci (GBS) are an important cause of serious neonatal illness (Anthony and Okada, 1977), which has been related to vaginal colonisation with these organisations (Ferrieri, Cleary and Seeds, 1977); several authors have reported the kinetics of the binding reaction between isolated vaginal epithelial cells and GBS (Mårdh and Weström, 1976; Zawaneh *et al.*, 1979). It has also been shown that the degree of attachment between bacteria and vaginal cells is related to the hormonal status of the vaginal-cell donor, reaching a maximum near the time of ovulation (Botta, 1979). At present, little is known of the particular bacterial cell-surface components concerned in the binding of GBS to epithelial cells. In group-A streptococci, membrane lipoteichoic acid (LTA) at the surface of the bacteria, is responsible, at least in part, for their adherence to buccal epithelial cells (BEC) (Beachey, 1975). This paper describes features of the binding of GBS to BEC and indicates the nature of the adhesin on the GBS cell surface that mediates attachment of the organism to BEC.

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MATERIALS AND METHODS

Bacteria. Type-III GBS (strain NCTC11080) and group-A streptococcus (strain NCTC10085) were from the National Collection of Type Cultures, Colindale Avenue, London NW9 5HT and were grown in static culture for 18 h at 37°C in Todd-Hewitt Broth (Oxoid CM189).

Buccal cells were gently scraped with a wooden spatula from the inside of the cheek of a healthy male donor and suspended in 10 ml of Dulbecco's Phosphate-buffered Saline (PBS; Oxoid BR14a). The suspension was placed in a thin-walled test tube and treated in a sonic water bath (Model 6441A, Dawe Inst. Ltd, Concord Road, Western Avenue, London W3 0SD) for $1\frac{1}{2}$ min to aid the removal of background commensal organisms. The cells were then sedimented at 200 *g* for 10 min and washed twice more in PBS. The washed pellet was suspended in 2 ml of PBS and a cell count performed in a Neubauer counting chamber. The suspension was finally adjusted to a concentration of $2–6 \times 10^4$ cells/ml by dilution with PBS.

Adherence assay. The extinction at 650 nm (E_{650}) of the 18-h culture of GBS was adjusted to 0·43 which, from a previously determined calibration curve, yielded 10^8 cfu/ml. The volume was noted. The bacteria were sedimented at 900 *g* for 20 min, washed once in PBS, then resuspended to 10^8 cfu/ml in the previously noted volume of Dulbecco's PBS + Dulbecco "B" solution (Oxoid SR39) 0·5 ml/100 ml of PBS.

Samples of bacterial suspension (1 ml) were mixed with equal volumes of buccal-cell suspension in small, flat-bottomed sample tubes; the stoppered tubes were rotated at 30 rpm at 37°C for 45 min. After incubation, each mixture was filtered through a 10-μm filter (Gelman Sciences Inc., 600 S. Wagner Road, Ann Arbor, Michigan; polypropylene filter, 13-mm diameter) held in a Millipore filter holder, mounted on the end of a 10-ml syringe. Each filter was washed with 30 ml of PBS, carefully removed from the filter holder and inverted onto a drop of PBS on a clean microscope slide. The filters were lifted off the slide after approximately 2 min. The slides were air-dried, fixed in methanol for 5 min, and finally stained by Gram's method. The stained smears were mounted in DPX (dibutyl thallate xylol) under coverslips.

Each mixture of bacteria and buccal cells was prepared in duplicate and the results were averaged. Counting was performed at a magnification of 400 and for each experiment the bacteria adherent to each of 100 separate buccal cells was counted. Statistical significance was tested by Student's *t* test.

Preparation of sonicate from GBS. GBS were grown for 18 h in 3 L of Todd-Hewitt broth, at 37°C. Bacteria were harvested by centrifugation at 20 000 *g* for 10 min, washed once in PBS (*p*H 7·4, 0·01M) and suspended in 20 ml of PBS. The resulting suspension was treated in 5-ml portions in the sonic waterbath for 5 min and the cells were then sedimented at 20 000 *g* for 10 min. The supernate was collected and dialysed against 0·005M tris-HCl buffer (*p*H 7·4) for $3\frac{1}{2}$ h at 4°C. The material was then lyophilised.

Heat-treated sonicate was prepared by autoclaving at 121°C for 15 min. For periodate oxidation of the sonicate, a 2-ml sample (protein 140 μg/ml in 0·01M PBS, *p*H 7·4) was made 0·1M in sodium metaperiodate and left overnight at room temperature, in the dark. Excess periodate was destroyed by treatment with ethylene glycol, followed by lyophilisation. The sample was then re-dissolved in 2 ml of distilled water. A control sample was treated in the same way except that periodate was not added.

Preparation of membrane lipoteichoic acid. LTA was extracted from the cytoplasmic membrane of type-III GBS with cold 80% aqueous phenol and purified on a column of Sepharose 6B by the method of Coley, Duckworth and Baddiley (1975).

Pretreatment of GBS. Bacterial suspensions were treated with periodate for 5 min at room temperature in 4 ml of acetate buffer, *p*H 4·6, containing sodium meta-periodate 10 mg/ml. Control organisms were suspended in the same buffer but which did not contain periodate. The bacteria were then washed once in PBS before being resuspended in Dulbecco's PBS + "B" to the required concentration. Bacteria were either heated in a 75°C water bath for 30 min or in an autoclave to 121°C for 15 min.

Bacteria were treated with trypsin (E.C. 3,4,21,4, Type III Sigma Chemical Co.) at a concentration of 10 000 N-benzoyl-L-arginine ethyl ester units/ml in 46 mM tris-HCl (*p*H 7·5) containing 11·5 mM $CaCl_2$ for 1 h at 37°C by the method of Saunders and Miller (1980).

Pretreatment of bacteria with various sugars was by incubation, at 37°C, of washed cells with

the relevant sugar, dissolved in Dulbecco's PBS+"B". Bacteria were washed once in Dulbecco's PBS+"B" before the adherence assay.

Pretreatment of buccal cells. Washed BEC were treated with streptococcal sonicate by mixing the cells with the sonicate on a tube rotator at 37°C for 1 h. The buccal cells were then sedimented by centrifugation at 200 *g* for 10 min.

Buccal cells were treated with LTA 0·7 mg/ml in the same manner as was the sonicate. Periodate treatment of the buccal cells was performed in the same way as for the GBS.

Analytical procedures. Protein concentration was estimated by the method of Lowry *et al.* (1951).

Polyacrylamide-gel electrophoresis (PAGE) was done in the presence of 1% sodium dodecyl sulphate (SDS) on 10% polyacrylamide slab gels with the discontinuous buffer system of Laemmli and Favre (1973). The gels were stained for protein with Coomassie brilliant blue R. A mixture of reference proteins (BDH, Poole, Dorset BH12 4NN) containing cytochrome C (mol. wt 12 300), myoglobin (17 200), chymotrypsinogen A (25 700), ovalbumin (45 000), albumin (66 200) and ovotransferrin (76–78 000) was used as an indicator of molecular weights.

Results

In preliminary experiments to confirm that GBS would adhere to BEC, their adherence was compared with that of group-A streptococci, which previously have been shown to become bound to BEC (Alkan, Ofek and Beachey, 1977). There was no statistically significant difference ($p > 0{\cdot}1$) between the number of these two organisms binding to BEC (table I).

TABLE I

Adherence of group-A streptococci and type-III GBS to human BEC

Bacterial cells	Mean* number of bacteria attached per epithelial cell ±2 SEM
None (background)	2·3±0·6
Type-III GBS	13·9±2·5
Group-A streptococci	15·7±3·2

GBS = group-B streptococci; BEC = buccal epithelial cells.
* Of count on 100 epithelial cells.

The kinetics of the binding reaction between BEC and type-III GBS, determined by incubating mixtures of GBS and BEC for 5, 15, 25, 35, 45, 60 and 90 min respectively, are shown in fig. 1. Maximum adherence was reached after 45 min with no significant increase after incubation for 90 min.

Periodate treatment of BEC

Treatment of BEC with sodium meta-periodate caused a significant decrease ($p < 0{\cdot}001$) in the binding of type-III GBS to the cells (table II).

Pretreatment of bacteria

Periodate treatment of type-III GBS did not cause a decrease in binding to BEC. Mild heat treatment of bacteria, 75°C for 30 min, did not decrease the binding either but autoclaving at 121°C for 15 min reduced binding to a very low level; the difference

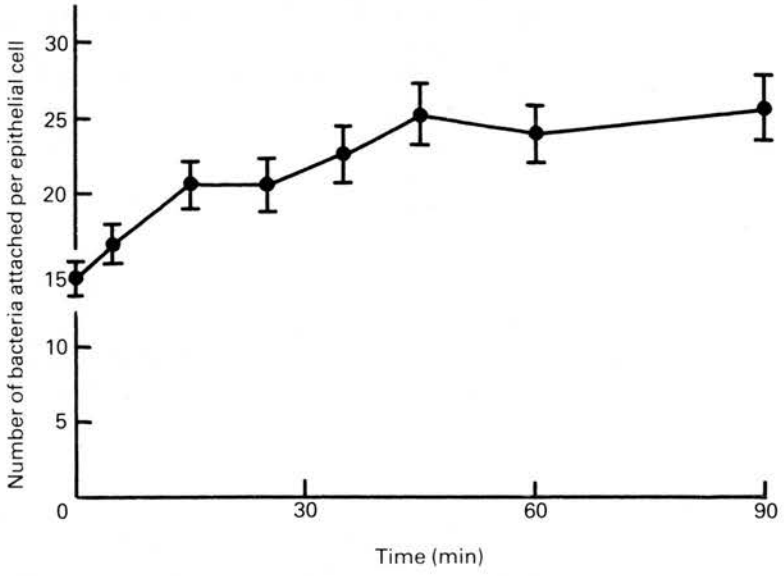

FIG. 1.—Effect of incubation time on adherence of type-III GBS to BEC. Each point represents the geometric mean (±1 SE) of results from 100 buccal cells.

was statistically highly significant ($p < 0{\cdot}001$). Treatment of GBS with trypsin caused a significant decrease ($p < 0{\cdot}01$) in their subsequent adherence to BEC. These results are shown in table III.

Blocking of adherence with sonicate from type-III GBS

Preincubation of BEC with the sonicate preparation blocked the binding of type-III GBS in a dose-response fashion (fig. 2).

Treatment of the sonicate at 121°C for 15 min significantly reduced its ability to block bacterial binding ($p < 0{\cdot}001$), but periodate oxidation of the sonicate affected its blocking capacity much less (table IV). Because there was no significant difference ($p > 0{\cdot}1$) between the activities of the periodate-treated sonicate and the periodate-control sonicate, it seems unlikely that a carbohydrate on the bacterial surface is concerned in adhesion.

TABLE II

Effect of periodate treatment of BEC on subsequent adherence of type-III GBS

Cells	Mean* number of bacteria attached per epithelial cell ±2 SEM
None (background)	10·8±1·9
Periodate-treated BEC (background)	10·1±1·6
BEC+GBS	19·3±2·8
Periodate-treated BEC+GBS	13·0±1·9

Footnotes as in table I.

TABLE III

Effect of pretreatment of type-III GBS on their adherence to BEC

Bacterial cells	Mean* number of bacteria attached per epithelial cell ±2 SEM
None (background)	19·9±3·4
GBS	26·5±3·2
Periodate-treated GBS	30·0±4·4
None (background)	21·4±3·2
GBS	30·5±3·4
Mildly heated† GBS	35·4±3·5
None (background)	19·2±2·6
GBS	30·5±3·4
Autoclaved‡ GBS	20·0±2·0
None (background)	24·5±3·2
GBS	40·8±5·9
Trypsinised GBS	30·8±3·6

† 75°C for 30 min.
‡ 121°C for 15 min.
Other footnotes as in table I.

Inhibition of adherence by sugars

Of all the sugars tested (0·2M D-galactose, 0·2M L-fucose, 0·05M lactose, 0·2M D-glucose, 0·2Mα-methyl-D-mannoside, 0·2M maltose, 0·1M N-acetyl-D-galactosamine and 0·1M N-acetyl-D-glucosamine), only N-acetyl-D-glucosamine produced a

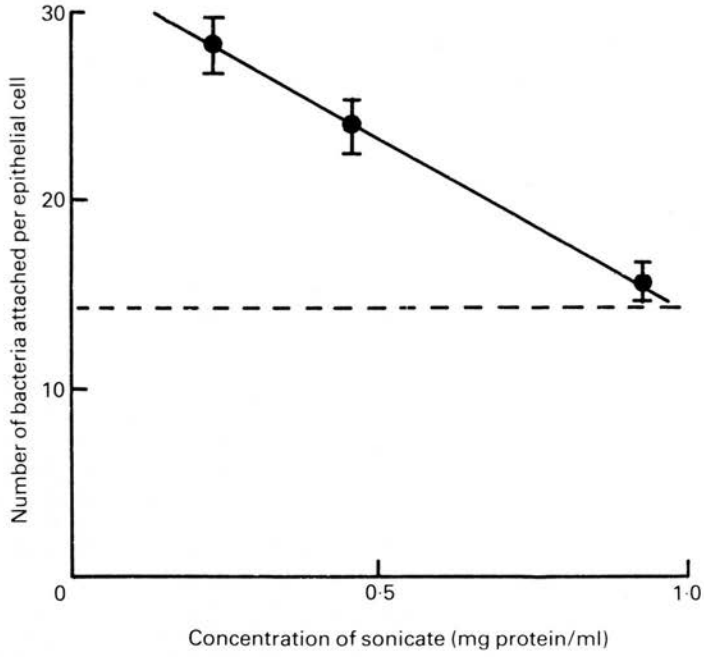

FIG. 2.—Dose-response curve of inhibition of binding by type-III GBS sonicate. Each point represents the geometric mean (±1 SE) of results from 100 buccal cells. – – – = background.

TABLE IV

Effect of heat and periodate oxidation on the capacity of type-III GBS sonicate to block binding of type-III GBS to BEC

Epithelial and bacterial cells	Mean* number of bacteria attached per epithelial cell ±2 SEM
Untreated BEC (background)	2·6±1·4
Untreated BEC+GBS	8·5±1·6
Sonicate-treated BEC+GBS	3·2±0·5
Heated† sonicate-treated BEC+GBS	6·7±0·9
Periodate-oxidised sonicate-treated BEC+GBS	5·0±0·8
Unoxidised control sonicate-treated BEC+GBS	4·2±1·1

† 121°C for 15 min.
Other footnotes as in table I.

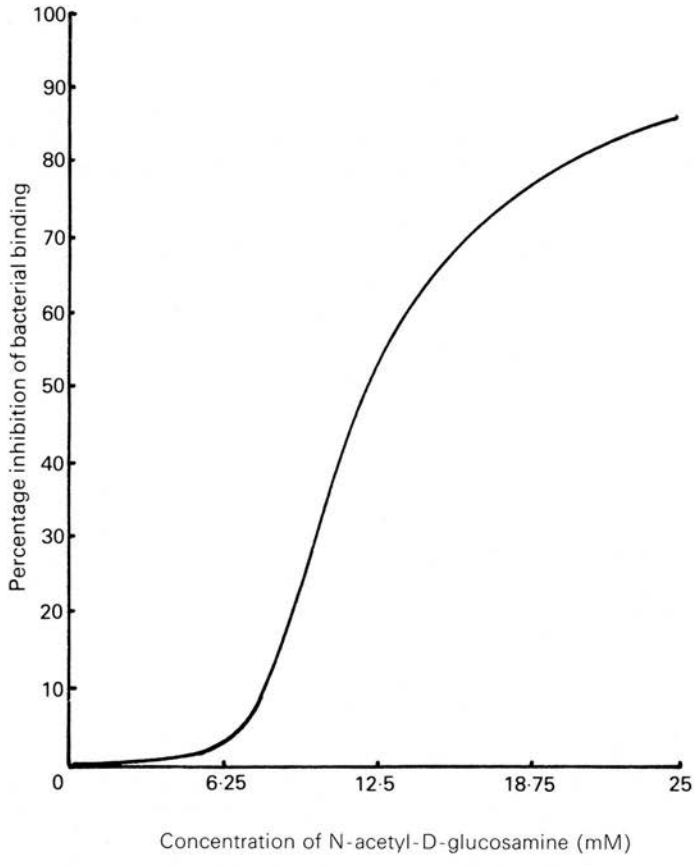

FIG. 3.—Dose-response curve of inhibition of binding of type-III GBS to BEC by N-acetyl-D-glucosamine. Means of three counts.

TABLE V

Effect of pretreatment of BEC with type-III GBS membrane LTA on subsequent adherence of type III-GBS

Epithelial and bacterial cells	Mean* number of bacteria attached per epithelial cell ±2 SEM
Untreated BEC (background)	22·2±2·6
LTA-treated BEC+GBS	28·9±3·8
Untreated BEC+GBS	33·0±3·2

LTA = lipoteichoic acid.
Other footnotes as in table I.

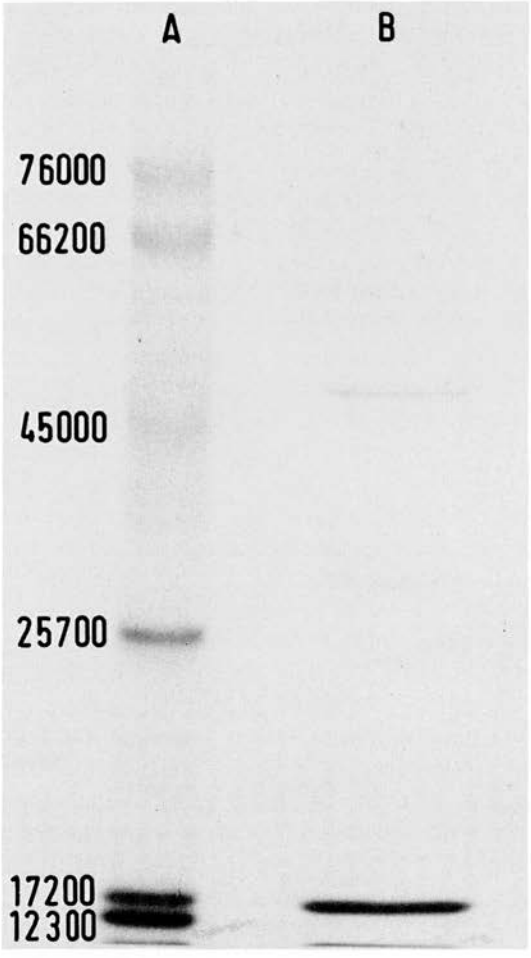

FIG. 4.—SDS-polyacrylamide-gel electrophoresis of sonicate preparation from type-III GBS. Lane A, molecular-weight markers; lane B, sonicate from type-III GBS.

significant decrease ($p < 0{\cdot}001$) in subsequent attachment of type-III GBS to BEC. A dose-response curve is shown in fig. 3.

Pretreatment of BEC with membrane LTA

No significant difference in binding was evident between treated and untreated cells ($p > 0{\cdot}05$) (table V).

Polyacrylamide-gel electrophoresis

SDS-PAGE of the sonicate preparation revealed several protein bands, with one major band of low molecular weight (*c.* 17 000) as shown in fig. 4.

Discussion

Buccal epithelial cells have been widely used as target cells in experiments designed to study the adherence of many different bacteria to epithelial surfaces (Alkan, Ofek and Beachey, 1977; Atkinson and Trust, 1980; Craven *et al.*, 1980; Saunders and Miller, 1980; Yamazaki, Ebisu and Okada, 1981). Although a large proportion of buccal cells are non-viable, as judged by trypan-blue exclusion, several layers of dead cells are present in the buccal mucosa (Beachey, 1981). In view of this, it seems appropriate to use them as a target cell for in-vitro assays. The mechanism of attachment of group-A streptococci has been studied with the help of BEC-attachment assays (Beachey, 1975) and it was found that, in the assay described in this paper, group-A streptococci and type-III GBS were bound to BEC to the same extent.

Sodium meta-periodate cleaves the C-C bond between vicinal hydroxyl groups of sugars and when BEC were treated with periodate there was a highly significant drop in the number of GBS that adhered to the cells. Conversely, treatment of the GBS with periodate did not reduce the subsequent binding, suggesting that a sugar molecule on the surface of the buccal cells plays a role in the binding mechanism. Inhibition studies with various simple sugars indicate that N-acetyl-D-glucosamine may be concerned in this.

Mild heat treatment of the bacteria did not reduce binding but heating at 121°C for 15 min markedly reduced the degree of attachment; in both instances the bacteria were killed. Possibly the adhesin on the bacterial cell wall is a lectin, a thermostable protein not affected by a temperature of 75°C but denatured at 121°C.

Results obtained with the sonicate from type-III GBS also support the view that a protein rather than a carbohydrate on the bacterial cell surface is the relevant adhesin. Heat treatment of the sonicate greatly reduced its efficiency as a blocking agent whereas the periodate had very little, if any, effect. SDS-PAGE of the sonicate preparation indicated its heterogeneity although there was one dominant protein band of low molecular weight (c. 17 000). It now seems likely that many bacteria, including such diverse organisms as *Escherichia coli* (Ofek, Beachey and Sharon, 1978), *Eikenella corrodens* (Yamazaki *et al.*, 1981), *Aeromonas hydrophila* (Atkinson and Trust, 1980) and *Fusobacterium nucleatum* (Mongiello and Falkler, 1979) rely on lectin-type interactions for their adherence to mucosal surfaces. Work in our laboratories has also shown that *Corynebacterium parvum* may attach to macrophages through a lectin-like binding site in the cell wall of the bacterium (Bagg, Poxton and Weir, 1981).

The suggestion (Botta, 1979) that some characteristics of adhesion of GBS to human vaginal epithelial cells may be explained by a lectin-type interaction, is supported by our findings. Work recently published (Elbein *et al.*, 1981) describing the binding of GBS to influenza viral glycoproteins that have been inserted into the cell membrane of infected MDCK cells, also supports this view. In the system that we studied, in which type-III GBS attach to BEC, it seems that an N-acetyl-D-glucosamine specific lectin on the bacterial cell surface is involved in attachment of the organism to the eukaryotic cell.

Beachey (1975) has identified membrane LTA as being responsible for binding of group-A streptococci to epithelial cells. Preincubation of BEC with LTA extracted from the cytoplasmic membrane of type-III GBS failed to block significantly the attachment of GBS, so that it seems that the mechanism of their adherence differs from that of the group-A streptococci. This agrees with the finding that loss of LTA from GBS cell walls, caused by treatment with penicillin, did not inhibit adherence of the bacteria to vaginal cells (Zawaneh *et al.*, 1979).

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Comparison of Cell Surface Antigen Extracts From Two Serotypes of *Pasteurella haemolytica*

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Cells of *Pasteurella haemolytica* serotypes A1 and A6 were extracted with sodium salicylate and the chemical and antigenic composition of both extracts determined. The extracts were concentrated by ultrafiltration and the serotype antigen, measured by the indirect haemagglutination test, was estimated to have a molecular weight between 100000 and 300000. The chemical composition of sodium salicylate extracts (SSEs) from both serotypes was similar, having protein, carbohydrate, fatty acid and phosphorus present in the ratio 10:1:0·5:0·1. SDS-PAGE of both SSEs gave similar profiles with at least 48 bands present. These results suggest that sodium salicylate removes the outer membrane of *P. haemolytica*. Crossed immunoelectrophoresis indicated that a major serotype-specific antigen was present in SSEs of both strains. This antigen was extracted from the SSE with hot phenol/water and analysed by gas chromatography. The sugar composition of A1 and A6 phenol/water extract (PWE) was qualitatively identical although some differences in proportions were observed. A1 and A6 PWE antigens protected mice against homologous serotype challenge and A6 PWE protected against heterologous (A1) challenge.

INTRODUCTION

Pasteurella haemolytica is the causal organism of enzootic pneumonia and septicaemia in sheep. There are at least 15 serotypes of *P. haemolytica* (Fraser *et al.*, 1982) and these fall into the two biotypes described by Smith (1961).

Antigens from a number of A biotype strains have been obtained by extraction of cells with sodium salicylate (Gilmour *et al.*, 1979) and vaccines containing these antigens have been shown to confer protection against homologous but not heterologous serotype challenge in sheep (Gilmour *et al.*, 1979, 1983) and in mice (Evans & Wells, 1979).

Burrells *et al.* (1983) reported considerable cross-reactivity in ELISA between sodium salicylate-extracted antigens from different serotypes and Donachie *et al.* (1983), using the same method, demonstrated that serotype specificity resided in the polysaccharide antigens. Apart from these reports very little is known about the chemical, physical and antigenic composition of sodium salicylate extracts.

We describe here the results of chemical and *in vitro* immunological analyses of sodium salicylate extracts from *P. haemolytica* serotypes A1 and A6.

METHODS

Bacteria. Pasteurella haemolytica biotype A, serotypes 1 and 6 (A1 and A6), strains FA1 and FA6, were isolates from sheep with pneumonic pasteurellosis. They were stored lyophilized or in nutrient broth at −70 °C.

Abbreviations: IHA, indirect haemagglutination; KDO, ketodeoxyoctonic acid; PWE, phenol/water extract; SSE, sodium salicylate extract.

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Preparation of sodium salicylate extracts (*SSEs*). Strains of *P. haemolytica* A1 and A6 were removed from storage, checked for purity and serotype identity and inoculated into 50 ml Oxoid no. 2 broth. After incubation at 37 °C for 18 h with agitation, 15 ml of the resultant culture were inoculated into 1·5 l of the same medium and further incubated at 37 °C for 6 h with agitation. Optical density measurements were made with a nephelometer, previously correlated with viable counts, to check that growth had reached approximately 10^9 live bacteria ml^{-1}. The bacteria were sedimented by centrifugation at 4200 ***g*** for 40 min at 4 °C and resuspended in one tenth of the broth culture volume in 1 M-sodium salicylate. This suspension was shaken at 37 °C for 3 h and the bacteria were removed by centrifugation at 28000 ***g*** for 40 min at 4 °C. The clear supernate was dialysed against a dilute phosphate/saline buffer (PBS: 0·02 M-sodium phosphate, 0·03 M-sodium chloride; pH 7·6) for 48 h. It was then concentrated by ultrafiltration through Diaflo (Amicon, Lexington, Mass., USA) XM300 and XM100A membranes and any slight sediment removed by centrifugation. From a 3 l culture approximately 30 ml of an opalescent and slightly viscous concentrate was obtained. All concentration procedures were carried out at 4 °C and the final concentrated extract stored at − 20 °C or lyophilized. In later preparations the SSE was concentrated before dialysis without any apparent effect on the final product.

Preparation of phenol/water extracts (*PWEs*). SSEs were further extracted with phenol by a method similar to that described by Westphal *et al.* (1952). SSE (20 mg) was suspended in 4 ml distilled water and added to an equal volume of 90% (v/v) phenol. The mixture was heated at 68 °C for 10 min with shaking, cooled in an ice bath and then centrifuged at 28000 ***g*** for 30 min. The aqueous layer was removed, dialysed against running tap water for 24 h and lyophilized.

Antisera. Antisera were raised against washed whole cells of *P. haemolytica* A1 and A6 in New Zealand White rabbits using the procedure described by Fraser *et al.* (1982).

Indirect haemagglutination (*IHA*) *test.* A sample of the concentrated SSE or PWE was diluted in PBS in 1·0 ml volumes. Ox red blood cells, fixed in 1% glutaraldehyde in PBS (v/v) (Shirai *et al.*, 1975), were added to the sample dilutions to give a final concentration of 0·5% (v/v) and the suspensions incubated at 37 °C for 30 min. The cells were washed three times in PBS and resuspended to their original 2·0 ml volumes. A homologous rabbit antiserum at a previously determined optimal dilution was dispensed in 25 µl volumes into the wells of a U-bottomed microtitre plate (Cookes Engineering Co., Alexandria, Va., USA). After addition of 25 µl sensitized cell suspension per well the plate was allowed to stand at room temperature for 2 h and examined for haemagglutination. Positive and negative controls were included. IHA titres were expressed as reciprocals of the highest dilution of SSE or PWE which produced haemagglutination.

Crossed immunoelectrophoresis (*CIE*). The procedure was essentially that described by Weeke (1973*a*). The SSE and PWE antigens were examined by CIE on 5 × 5 cm squares of Gelbond film (FMC Corporation, Marine Colloids Division, Bio Products, Rockland, Me., USA) using barbital/glycine/Tris buffer pH 8·8 (Weeke, 1973*b*). Buffer was used undiluted (ionic strength 0·08) in the electrode reservoirs, but in gels and in sample buffer it was diluted 1 in 4 with distilled water. Electrophoresis was at 20 V cm^{-1} for 45 min in the first dimension and 4 V cm^{-1} for 18 h in the second. Gels were pressed, washed and stained with Coomassie blue as described by Weeke (1973*b*).

Chemical assays. Total dry weight of the SSE and PWE was determined by freeze drying after complete dialysis against water. Total protein was estimated by the Lowry method with bovine serum albumin as standard. Total carbohydrate was determined by the phenol/sulphuric acid method described by Dubois *et al.* (1956) with glucose as standard. Total phosphorus was determined by the method of Chen *et al.* (1956). For the estimation of fatty acid content freeze-dried samples of SSE (10 mg) were hydrolysed with 6 M-HCl (2 ml) at 105 °C for 4 h. The hydrolysates were then repeatedly extracted with diethyl ether (Clarke *et al.*, 1967), the residue dissolved in chloroform, and the fatty acid content determined by the copper colorimetric method of Duncombe (1963) with palmitic acid as standard.

Ketodeoxyoctonic acid (KDO) and heptose were measured by the method of Osborn (1963).

Vaccines. Aqueous suspensions of SSE (1·18 mg ml^{-1}) and PWE (0·44 mg ml^{-1}) of A1 and A6 were emulsified with equal volumes of Freund's complete adjuvant (Difco).

Vaccination and challenge. Six-week-old C57 black mice randomly allocated to groups of ten, were inoculated subcutaneously with 0·1 ml vaccine at day 0 and 14. For each serotype, vaccinated and unvaccinated groups of mice were challenged at 28 d after the initial vaccination according to the method of Evans & Wells (1979), except that no mice were killed immediately after challenge. Briefly, mice were challenged intraperitoneally with 0·5 ml of a mixture of bacterial cell suspension in mucin (1:4, v/v). The challenge doses for A1 and A6 were $1·3 \times 10^6$ colony forming units (c.f.u.) ml^{-1} and $2·9 \times 10^5$ c.f.u. ml^{-1} respectively. The mice were killed 6 h after challenge, their livers removed and the number of bacteria in each liver assessed by viable counting (Miles *et al.*, 1938). Counts from the livers of vaccinated and control mice were compared by the Mann–Whitney ranking test (Snedecor & Cochran, 1967).

Gas chromatography (*GC*). PWEs were hydrolysed with 2 M-hydrochloric acid at 100 °C for 3 h. The monosaccharides were analysed as alditol acetates prepared by the method of Poxton & Cartmill (1982) on a Pye Unicam 104 chromatograph using a column containing 3% (w/w) OV-225.

SDS-PAGE. This was done on 10% (w/v) acrylamide slab gels with the buffer system of Laemmli (1970) by the method described by Poxton & Brown (1979).

RESULTS

SSE was prepared from *P. haemolytica* serotype A1 (strain FA1) and serotype A6 (strain FA6) by the ultrafiltration method. Antigenic activity for A1, estimated as the IHA titre against homologous rabbit antiserum, was determined at each step in the concentration procedure. Samples were titrated in serial doubling dilutions from 1 in 5 to 1 in 640 in 1 ml volumes of PBS. Loss of antigenic activity in sediments during clarification by centrifugation was less than 1% of the total activity. Table 1 shows the specific IHA titres of the dialysed SSE before ultrafiltration, the ultrafiltrate, and the concentrated preparation of two different A1 SSE preparations passed through Diaflo XM100A or XM300 membranes. Antigenic activity was almost completely retained by the XM100A filter but appreciable amounts of antigen passed through the XM300 filter.

Both A1 and A6 SSE had a high degree of antigenicity when titrated against their respective homologous antisera in the IHA test. At a concentration of 2·5 mg ml^{-1} the SSEs of A1 and A6 had titres of 1 in 1000 and 1 in 5000 respectively.

The chemical analyses for A1 and A6 SSEs (Table 2) indicate a strong similarity in chemical composition with both preparations containing protein, carbohydrate, fatty acid and phosphorus in roughly the same ratio (10:1:0·5:0·1).

The antigens of *P. haemolytica* A1 and A6 SSE were visualized by CIE. The homologous antibody/antigen reactions for A1 and A6 SSE were similar in that one major antigen was present in each of the SSEs (Fig. 1 *a*, *b*). When the loading of the A6 SSE was increased to that of the A1 SSE (50 μg) three other precipitin lines were observed in the homologous antiserum gel. However, this resulted in a very diffuse and distorted precipitin line for the major antigen (result not shown). SSE antigens were also run in CIE with an intermediate gel containing heterologous antiserum between the antigen and the homologous antiserum-loaded gel. The major antigen for both SSEs passed through the intermediate gel and precipitated only in the homologous antiserum gel (Fig. 1 *c*, *d*). The major antigens would appear to be serotype-specific. When A6

Table 1. *IHA titres of two different SSE preparations from P. haemolytica A1 before and after ultrafiltration through XM300 and XM100A membranes*

Specific titre = titre/sample volume (ml); titres were determined as described in Methods. The XM300 and XM100A fractions were derived from different preparations.

	Specific titre of fraction	
Fraction	XM300 membrane	XM100A membrane
Dialysed SSE before ultrafiltration	0·39	2·6
Concentrate	100	>490
Ultrafiltrate	0·245	<0·021

Table 2. *Composition of sodium salicylate and phenol/water extract prepared from P. haemolytica serotypes A1 and A6*

Values are expressed as percentage of total dry weight; ND, not done.

	Serotype A1		Serotype A6	
Component	SSE	PWE	SSE	PWE
Phosphorus	0·4	1	1	2·8
Protein	57·9	6·3	56	0·4
Carbohydrate	6·0	14·8	5·4	12·4
Fatty acid	2·8	10·8	3·4	5·8
Heptose	ND	6·6	ND	2·4
KDO	ND	0·6	ND	0·01

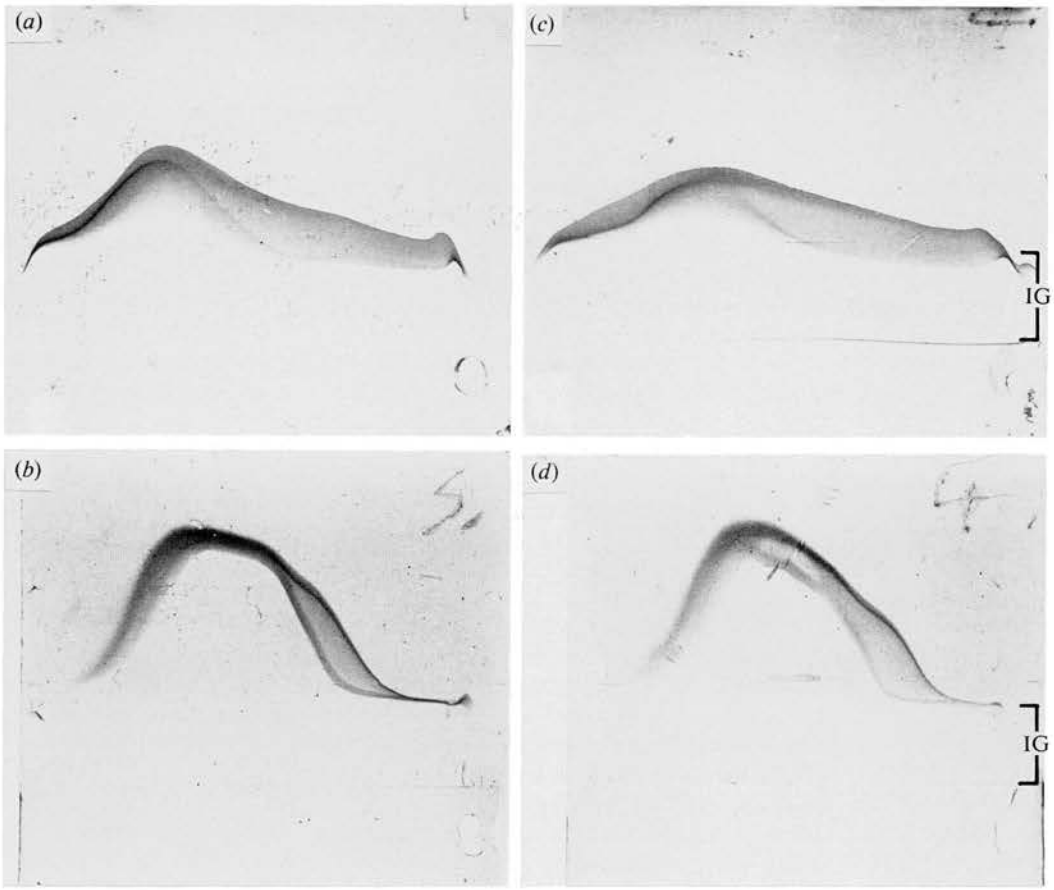

Fig. 1. CIE of (*a*) A1 SSE (50 μg) run against 100 μl A1 antiserum, with blank intermediate gel (IG); (*b*) A6 SSE (12·5 μg) run against 100 μl A6 antiserum with blank IG. CIE patterns obtained with (*c*) A1 SSE (50 μg) run against 100 μl A1 antiserum with IG containing 50 μl A6 antiserum; (*d*) A6 SSE (12·5 μg) run against 100 μl A6 antiserum with IG containing 50 μl A1 antiserum.

SSE at an equivalent loading to A1 SSE was run with an intermediate gel the three minor antigens were held back by the intermediate gel indicating that these antigens are common to A1 and A6 (result not shown).

The PWEs of both A1 and A6 had comparable levels of antigenicity when measured by IHA (1 in 1000 and 1 in 2000 respectively at concentrations of 1 mg ml^{-1}).

Both A1 and A6 PWEs were rich in polysaccharide and had little protein compared to their respective SSEs (Table 2). Both contained heptose and small amounts of KDO. When the PWEs of A1 and A6 were examined by GC the sugar compositions were similar, with glucose, galactose, mannose, glucosamine, galactosamine and two presumptive heptoses present in the molar ratios 1·8:1·5:0·15:1·0:1·5:1·5:2·4 for A1 PWE and 3·3:1·3:2·1:1·0:0·4:0·4:0·35 for A6 PWE. The presumptive heptoses were later confirmed by mass spectrometry.

PWEs of SSE were also run on CIE. The antigenic profiles obtained when these preparations were run through a blank intermediate gel into a homologous antiserum gel were similar to those found for the SSE preparations. One major peak was visible for each serotype (Fig. 2*a*, *b*). In addition two minor peaks were present in the intermediate gel of the A1 PWE run and one minor peak was visible at the interface of the two gels in the A6 PWE run. When the intermediate gel contained heterologous antiserum the major antigens of both serotypes were unaffected and precipitated in the homologous antiserum gel (Fig. 2*c*, *d*). One of the two minor

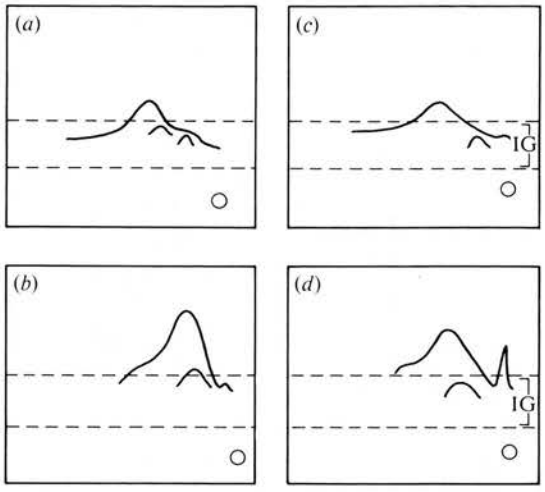

Fig. 2. CIE of (*a*) A1 PWE (10 μg) run against 100 μl A1 antiserum, with blank intermediate gel (IG); (*b*) A6 PWE (10 μg) run against 100 μl A6 antiserum with blank IG; (*c*) A1 PWE (10 μg) run against 100 μl A1 antiserum with IG containing 50 μl A6 antiserum and (*d*) A6 PWE (10 μg) run against 100 μl A6 antiserum with IG containing 50 μl A1 antiserum.

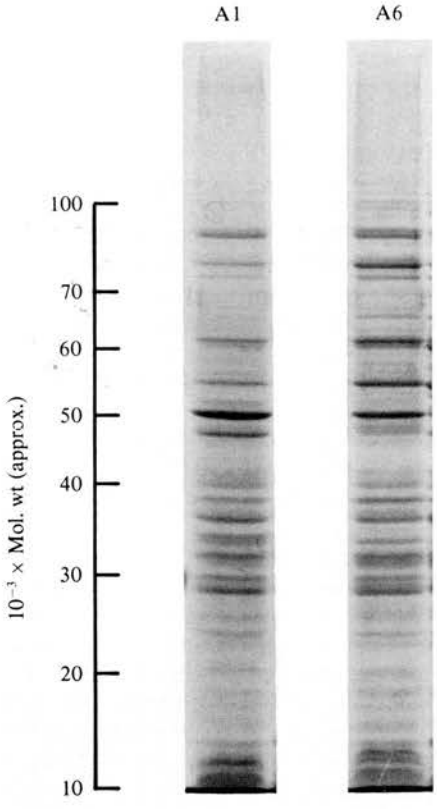

Fig. 3. SDS-PAGE of sodium salicylate extracts (300 μg) of *P. haemolytica* serotypes A1 and A6 on 10% acrylamide gels stained with Coomassie blue.

Table 3. *Viable counts of P. haemolytica A1 or A6 in the livers of control mice and mice vaccinated with SSE or PWE from P. haemolytica A1 or A6*

Vaccine	Challenge (dose: c.f.u. ml^{-1})	log_{10} Viable count	
		Control	Vaccinated
A1 SSE	A1 ($1{\cdot}3\times10^6$)	$6{\cdot}68\pm1{\cdot}7$	$3{\cdot}4\pm0{\cdot}9$*
A1 PWE	A1 ($1{\cdot}3\times10^6$)	$6{\cdot}68\pm1{\cdot}7$	$4{\cdot}9\pm1{\cdot}8$†
A6 PWE	A1 ($1{\cdot}3\times10^6$)	$6{\cdot}68\pm1{\cdot}7$	$5{\cdot}1\pm1{\cdot}9$‡
A6 SSE	A6 ($2{\cdot}9\times10^5$)	$7{\cdot}42\pm0{\cdot}4$	$2{\cdot}8\pm0{\cdot}3$*
A6 PWE	A6 ($2{\cdot}9\times10^5$)	$7{\cdot}42\pm0{\cdot}4$	$4{\cdot}5\pm1{\cdot}7$†
A1 PWE	A6 ($2{\cdot}9\times10^5$)	$7{\cdot}42\pm0{\cdot}4$	$6{\cdot}7\pm0{\cdot}9$

* Significant at 0·001 level (Mann–Whitney ranking test).
† Significant at 0·01 level.
‡ Significant at 0·05 level.

antigens of A1 PWE was apparently unaffected and the other antigen failed to precipitate (Fig. 2*c*). The minor antigen present in A6 PWE was precipitated within the intermediate gel indicating some reaction with the heterologous antiserum (Fig. 2*a*).

Analysis of the proteins present in A1 and A6 SSE by SDS-PAGE (Fig. 3) showed that the two serotypes contained a similar pattern of polypeptides. At least 48 bands were present with one major band at around 51000.

Table 3 shows that PWE from both serotypes conferred significant protection on mice challenged with homologous serotype culture. When challenged with the heterologous serotype, A6 PWE protected against A1 ($P = 0{\cdot}05$) but A1 PWE did not protect against A6. The degree of protection against homologous serotype challenge afforded by SSE (significant at 0·001 level) in each case was greater than that of the PWE (both significant at 0·01 level).

DISCUSSION

Sodium salicylate may remove a whole complex of lipopolysaccharide (LPS) and protein from the outer surface of *P. haemolytica* cells. As the antigenic activity determined by IHA titres is high in the supernate after extraction and this activity is retained after ultrafiltration through an XM100A membrane but not an XM300 membrane (Table 1) it is possible to suggest an apparent molecular size of between 100000 and 300000 for the antigenic complex in the A1 and A6 serotypes. Chemical analyses of both SSEs showed them to be similar.

SDS-PAGE analysis of the SSEs further illustrates the close similarity of the two serotypes in structure as there are no major differences between them in the number or migration of polypeptide bands present. This contrasts with a previous study by Thompson & Mould (1975) where proteins extracted with phenol/acetic acid and water from the two serotypes produced different profiles on electrophoresis in polyacrylamide gels.

The profiles obtained by SDS-PAGE indicate that the SSE of both serotypes contains a large number of proteins. The exact origin of these proteins is unknown but it is likely that outer membrane proteins are present.

CIE of the SSEs from A1 and A6 detected a number of antigens. In each case a dominant serotype-specific antigen was present and in A6 SSE three minor antigens which were also common to A1 were sometimes observed by precipitation with heterologous antiserum in an intermediate gel. The serotype-specific antigen, as detected by CIE and IHA, of each serotype was extractable with hot phenol/water and shown to be largely polysaccharide. The presence of heptose and KDO, which are generally regarded as markers for LPS, make it likely that the LPSs of *P. haemolytica* A1 and A6 are present in the PWE preparations and visualized in CIE. The identical monosaccharide composition of A1 and A6 PWEs revealed by GC suggests that the antigenic differences seen in CIE and IHA are a result of either the linkages between sugars or the proportion of sugars in the polysaccharides, and are not due to completely different components.

However, CIE indicated that the PWEs were not pure preparations of the serotype antigens as, for each serotype, minor antigenic peaks were held back in intermediate gels containing heterologous antiserum. These cross-reacting antigens may be present in the small amount of contaminating protein or may be distinct polysaccharide components. Further purification of the serotype antigen with immunosorbent techniques has so far been unsuccessful.

The finding that the PWEs of both A1 and A6 were protective in mice against homologous challenge and, in the case of A6 PWE, against heterologous challenge indicates that the serotype-specific protection reported for SSE vaccines by Evans & Wells (1979) probably resides in the serotype antigen extracted by the phenol/water. The one-way cross-protection seen with A6 PWE against A1 challenge could be explained by the presence of antigenic determinants within A6 PWE which are shared with A1 PWE and important in protection against A1. Other determinants which are not shared may be important in protection against A6.

The degree of protection afforded by PWE was less than that provided by SSE which may indicate that the immunogenicity of the serotype antigen is enhanced by close association with other outer membrane components such as proteins. Karch & Nixdorff (1981) have shown that a combination of outer membrane protein and LPS from *Proteus mirabilis* gave rise to increased immune responses to both components compared to those obtained when they were injected individually into mice.

The possibility that antigens other than the serotype antigens are important in protection is supported by the work of Biberstein & Thompson (1965) and Knight *et al.* (1969) who showed that cross-protection unrelated to the serotype antigen does occur between *P. haemolytica* serotypes. Non-specific protection against *Salmonella typhimurium* by rough LPS preparations of *P. haemolytica* A7 has been reported by Rimsay *et al.* (1981). On the other hand, available evidence suggests that, in sheep, protection is serotype-specific (Gilmour *et al.*, 1979).

If the components involved in the immune response to *P. haemolytica* can be identified and characterized it may be possible to select strains which produce a more effective antigenic complex for inclusion in pasteurella vaccines.

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Identification of non-haemolytic pasteurellae cultured from the lungs of cattle

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PASTEURELLA multocida is the cause of haemorrhagic septicaemia of cattle and buffalo in the tropics and subtropics (Bain 1963) and is one of the causes of respiratory disease of cattle throughout the world (Jensen and others 1976, Gilmour 1978).

Carter (1963) demonstrated four serological types designated A, B, D and E in an indirect haemagglutination (IHA) test. Serotype A is the most common isolate from mammals and birds (Carter 1957, Perreau and others 1962) and can be distinguished from the other varieties of *P multocida* by the hyaluronidase decapsulation (HYD) test (Carter 1955, 1972).

Madsen and others (1985) reported certain mucoid *P multocida*-like organisms (Taxon 13) which are also HYD test positive. However, unlike *P multocida*, these organisms do not produce indole and do not convert mannitol and sorbitol to acid.

This investigation sought to use the API20E and conventional tests to identify presumptive *P multocida* strains isolated from pneumonic lungs of cattle.

The 93 presumptive *P multocida* strains used in this study were sent to Moredun Research Institute by VICS throughout the UK. They were all non-haemolytic thus excluding *P haemolytica*. Each isolate was subcultured on dextrose starch agar (DSA) (Gibco Europe, Paisley, Scotland), harvested in skimmed milk and lyophilised.

Lyophilised cultures were reconstituted in brain heart infusion broth (BHIB) (Oxoid, Basingstoke, Hampshire) and subcultured on DSA plates that were then incubated at 37°C overnight. Three or four isolated colonies were picked with a sterile cotton wool swab, emulsified in 1 per cent yeast extract, and tested in the API20E system (API Laboratory Products, Basingstoke, Hampshire) according to the manufacturer's instructions.

Rabbit antiserum to *P multocida* type A was kindly supplied by Dr V. Norrung, Staatens Veterinaere Serum-laboratorium, Denmark.

Isolates were tested by IHA (Carter 1955, 1972), and by the HYD test using hyaluronidase-producing *Staphylococcus aureus* (Carter and Rundell 1975).

Of the 93 strains examined 37 were identified as *P multocida* in the API20E tests, and eight were *Pasteurella* species strains in that they produced indole in the API20E reactions. Seven strains conformed to Madsen's Taxon 13 classification on a combination of API20E and conventional biochemical tests. Forty-one did not fall into any of the above categories.

The results of the IHA and HYD tests are summarised in Table 1. Thirty-three of the 37 typical *P multocida* strains were classified as type A in the HYD test but only 21 on the IHA test. All seven Taxon 13 strains were HYD positive and 17 of 41 biochemically unidentified strains reacted to both tests for serotype A.

TABLE 1: Reaction of the isolates in the HYD and IHA tests

Biochemical test group	Number of strains	Number of positive strains HYD	IHA
Typical *P multocida*	37	33	21
Pasteurella species	8	0	0
Taxon 13	7	7	0
Unidentified *	41	17	17

* 17 of these strains were API20E negative but all reacted to both the HYD and IHA tests

The results indicate a diversity both in the strains of *P multocida* and in other pasteurella-like organisms recovered from cattle with respiratory disease. Their identification presents considerable problems to the clinical bacteriologist.

Seventeen of the IHA positive strains examined did not react in the API20E system, possibly because they were fastidious and the 1 per cent yeast extract was not rich enough to give sufficient growth (Oberhofer 1981, Porres and Porter 1975).

Madsen and others (1985) found during biochemical investigations of 61 isolates of *P multocida* from pneumonic calf lungs, that 67 per cent of the isolates in Denmark belonged to Taxon 13. In contrast, only 7·5 per cent of the strains examined by the present authors could be regarded as Taxon 13. The reason for this lower prevalence in the UK is unknown.

Only 38 of the 93 strains gave reactions with anti-A serum. The 38 positive strains comprised 21 of the 37 typical *P multocida* strains and 17 of the unidentified strains. It is likely that some of the non reactor strains had dissociated and lost their capsules during storage and would not be identified by the IHA test. Other strains could have belonged to the other Carter serotypes B, D and E. An interesting observation from this study was that none of the Taxon 13 strains was positive in the IHA test, although Taxon 13 strains have not previously been examined by this technique (Madsen and others 1985). In this study also, 57 of the 93 cultures examined were HYD positive including all the seven Taxon 13 strains. Like the IHA test, the HYD test can be used only to type mucoid, capsulated strains.

It is clear that a number of *P multocida*-like organisms are present in the respiratory tracts of cattle and further work is necessary to elucidate the role of such strains in respiratory infections. The results also highlight the need for a practical scheme of classification for the clinical veterinary bacteriologist.

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Short Communications

Use of the egg hatch assay on sheep faecal samples for the detection of benzimidazole resistant nematodes

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ANTHELMINTIC resistance in nematodes of grazing livestock is of increasing concern in a number of areas throughout the world, because genes for resistance are present in most of the important genera of nematodes that affect grazing animals. It is in countries where *Haemonchus contortus* of sheep and goats is endemic that the major problems with anthelmintic resistance are generally encountered.

Many reports of resistance involve the benzimidazole group of anthelmintics and several techniques have been developed to detect the presence of parasites resistant to this group. These techniques have been reviewed by Presidente (1985) and more recently by Taylor and Hunt (1989). The most widely used in vitro test for detecting benzimidazole resistance is the egg hatch assay. There are several variations of the assay but they are all based on the ovicidal properties of the benzimidazole drugs and the ability of eggs from resistant strains to develop and hatch at higher concentrations of the anthelmintic than their susceptible counterparts. The need for undeveloped eggs (Coles and Simpkin 1977) has been a major obstacle to the application of in vitro egg hatch assays in routine diagnosis.

Several techniques have been described to overcome the problems of using the egg hatch assay to screen for benzimidazole resistance in the field. One method described by Whitlock and others (1980) involved the recovery of nematode eggs by sugar flotation, and their incubation with a range of concentrations of anthelmintic in McCartney flasks before transportation back to the laboratory. A different approach was to prevent the development of nematode eggs in transit to the laboratory by storing faecal samples on ice (Smith-Buijs and Borgsteede 1986). This paper describes a simple anaerobic storage system that can be used for the submission of faecal samples from the field to the laboratory up to seven days after collection.

To provide the nematode eggs required for the development of storage techniques and for the egg hatch assays, parasite-free Dorset horn lambs were infected with third stage larvae of either benzimidazole resistant or susceptible strains of *H contortus* or *Ostertagia circumcincta*. The resistant strains were originally isolated from the field and maintained in the laboratory by passage through worm-free lambs. Anthelmintic sensitivity to benzimidazole anthelmintics had been previously determined by slaughter trials and egg hatch assay (Cawthorne and Whitehead 1983, Cawthorne and Cheong 1984). Susceptible strains had been maintained in the laboratory for over 20 years in a similar manner and had not experienced any anthelmintic selection pressure.

Faeces samples were examined from 21 days after infection for the presence of nematode eggs. When egg counts had risen above 500 epg, approximately 20 g of faeces were taken from each infected animal and divided into two subsamples, A and B. Sample A was screened immediately by using the egg hatch method described by Cawthorne and Whitehead (1983). Sample B was stored under anaerobic conditions for up to seven days at 27°C. The method of storage involved adding 10 g of faeces to approximately 90 ml of water in a 100 ml screw-top plastic bottle containing 10 glass balls. The bottle was shaken vigorously for one minute to break up the pellets and release the eggs into suspension, and then placed in an incubator at 27°C. Anaerobic conditions, verified with an oxygen meter (Camlab Schott-Gerate), developed within three hours.

Faecal samples were stored at 27°C for up to 14 days before the eggs were recovered and screened for benzimidazole resistance using the method described above. Seven days was the longest time that eggs could be stored before their mortality exceeded 5 per cent of the population.

TABLE 1: ED50 values (μg/ml) and resistance factors for resistant and susceptible strains of *Ostertagia circumcincta* (O/C) and *Haemonchus contortus* (H/C) after different storage times

	Susceptible strains	Resistant strains	Resistance factor
O/C Fresh	0·053	0·748	14·1
5 days	0·067	0·576	8·6
7 days	0·087	0·543	6·2
H/C Fresh	0·043	0·334	7·8
5 days	0·045	0·309	6·9
7 days	0·037	0·363	9·8

ED50 values (the concentration of thiabendazole in μg/ml required to kill 50 per cent of eggs) obtained from the in vitro egg hatch assays using three replicates of fresh and anaerobically stored samples are given in Table 1. The corresponding dose response lines for benzimidazole susceptible and resistant strains of *O circumcincta* and *H contortus* are shown in Figs 1 and 2, respectively.

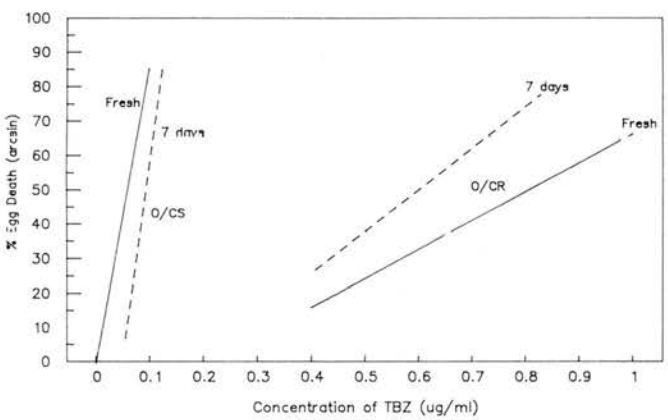

FIG 1: Comparative dose-response lines for fresh and anaerobically stored eggs of benzimidazole susceptible and resistant strains of *Ostertagia circumcincta*

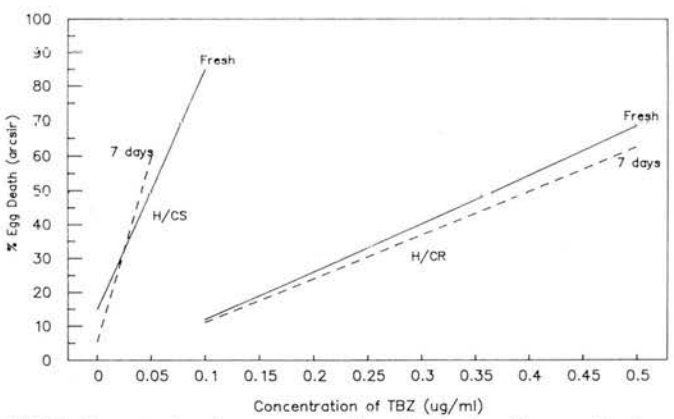

FIG 2: Comparative dose-response lines for fresh and anaerobically stored eggs of benzimidazole susceptible and resistant strains of *Haemonchus contortus*

The results demonstrate that the anaerobic storage of nematode eggs for up to seven days had no significant effect on ED50 values when compared with the values from fresh samples. Although there was some variation in ED50 values and the resistance factors with storage, they remained within the normal variations encountered with the egg hatch assay technique (Boersma 1983, Waller and Prichard 1986).

The use of this anaerobic storage method should make it possible to apply the egg hatch assay more widely for screening for benzimidazole resistance in the field.

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Outer membrane proteins of bovine strains of *Pasteurella multocida* type A and their doubtful role as protective antigens

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Summary. Outer membranes were prepared by the Sarkosyl method from 30 strains of *Pasteurella multocida* and the closely related Taxon 13, which had been isolated from cattle. The patterns of the outer membrane proteins (OMPs) on SDS-PAGE were generally similar to one another, though the four major proteins (*a–d*) varied somewhat in molecular mass; these patterns allowed the strains to be arranged into 12 groups. Taxon 13 strains and typical *P. multocida* strains were indistinguishable, both types being found within the same group. Mice were vaccinated with heat-killed bacteria of three strains and challenged with 10 LD50 of homologous and heterologous live bacteria, representing groups based on OMP patterns; the best protection was afforded by strain W674, which protected against nine of the 17 challenge strains; but there was no correlation between protection and PAGE pattern. Pre-vaccination and pre-challenge sera were used in immunoblotting to probe OMPs from protective and non-protective strains. All three vaccines produced antibody to proteins *a* and *d*; these proteins appeared to be common to all strains, varying in molecular mass but not in overall antigenic expression. The antibody response to the other two major OMPs appeared to be PAGE-group specific. There was no correlation between protection and the antigen pattern seen by immunoblotting.

Introduction

Pasteurella multocida is a small gram-negative, saccharolytic, indole-positive, facultatively anaerobic cocco-bacillus. Carter's capsular serotype A is associated with several animal diseases of worldwide importance such as bovine and porcine pneumonia, fowl cholera and rabbit septicaemia.[1–3] Type A can be distinguished from other types[4,5] by indirect haemagglutination (IHA) and hyaluronidase decapsulation (HYD). Similar organisms[6] that do not produce indole and are unable to ferment mannitol and sorbitol have been designated Taxon 13.

Many studies of the pathogenic mechanisms and immunogenicity of surface extracts of *P. multocida* serotypes have been made with the aim of developing vaccines.[7–11] However, effective vaccines have been developed only for types B and E, the haemorrhagic septicaemia serotypes.[12] Lugtenburg *et al.*[13,14] have shown a correlation between cell-envelope-protein type and pathogenicity of strains, presumably type D,[15] isolated from swine atrophic rhinitis. For type A strains, an experimental vaccine for rabbits has been reported recently. It is based on a potassium thiocyanate extract of whole bacteria[16] and the protective immunogen has been identified as a 37·5-Kda outer membrane protein (OMP).[17]

The aim of this study was to analyse the OMP profiles of *P. multocida* type A and the related Taxon 13 strains isolated from bovine pneumonia, to determine the heterogeneity of this group, and to attempt to define a protective immunogen in a mouse septicaemia model, by investigation of the immunogenicity of the OMPs.

Materials and methods

Bacteria

With the exception of the reference strain NCTC 10322 which was obtained from the National Collection of Type Cultures, London NW9 5HT, all the strains were isolated at Veterinary Investigation Centres from the lungs of calves with pneumonia, and were identified

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provisionally as *P. multocida*. They were sent to the Moredun Research Institute (MRI) on blood-agar plates, and subcultured there once on plates of Dextrose Starch Agar (DSA; Gibco). After overnight incubation, pure cultures were suspended in skimmed milk and stored by freeze drying. Strains were typed by IHA and HYD,[20] and identified further by biochemical reactions in a combination of conventional tube tests and API 20E test strips. The IHA technique was similar to that of Carter,[4, 18] except that glutaraldehyde-fixed sheep erythrocytes were used instead of human group O cells.[19] Tests were performed in 50-μl volumes in U-shaped wells of microtitration plates (Sterilin) with rabbit antiserum kindly supplied by Dr V. Norring (Statens Veterinaire Serum Laboratorium, Denmark). From the 93 strains submitted, 30 were selected for this study: 24 of *P. multocida* type A, and six of Taxon 13. More details of the bacterial identification have been published elsewhere.[21]

Vaccine strains and preparation of vaccine

In a preliminary study,[22] vaccine preparations from nine strains, containing capsule, lipopolysaccharide, formalin-killed bacteria or heat-killed bacteria, were tested for protective ability against homologous and heterologous challenge with 10 LD50 of live bacteria. The heat-killed cell vaccines of *P. multocida* strains W674 and W829 afforded the best heterologous protection. These two strains, and a Taxon 13 strain (X120) which gave no protection, were selected for the present study.

Preparation of vaccine. Freeze-dried bacteria were plated on DSA and incubated overnight at 37°C in a moist sealed chamber to enhance hyaluronic acid production.[23] Several iridescent colonies, which represent the wild-type (virulent) phenotype, were inoculated into 30 ml of Brain Heart Infusion Broth (BHIB; Oxoid) and incubated overnight at 37°C in an orbital shaker. The culture was transferred to 1 L of BHIB in a 2-L conical flask and kept for 6 h at 37°C in an orbital incubator. The cells were harvested by centrifugation at 12 000 *g* for 20 min at 20°C, washed once in phosphate buffered saline pH 7·4 (PBS) and finally resuspended in 10 ml of PBS. They were killed in a water bath at 68°C for 90 min and, after sampling for sterility, they were freeze-dried. Vaccine consisted of dried bacteria (final concentration 5 mg/ml) in equal volumes of water and Bayol F (Esso) containing Arlacel (Sigma) 10%.

Immunisation and challenge of mice

Swiss white mice of both sexes, bred at MRI, were 4–6 weeks old at the beginning of an experiment. Groups of 10 mice were given two doses of 0·2 ml of vaccine subcutaneously, 14 days apart. Vaccinated mice and non-vaccinated controls were challenged intraperitoneally with 10 LD50 of a 6-h broth culture of the organism, 14 days after the second vaccination; and deaths were recorded up to 48 h after challenge. The LD50 was determined previously by the method of Reed and Muench;[24] it ranged from $7{\cdot}5 \times 10^6$ to $1{\cdot}2 \times 10^8$ organisms.

A 50-μl sample of blood was collected from the tail vein of each mouse pre-vaccination and pre-challenge. The blood from each group was pooled, and the serum was stored at −20°C.

Isolation of outer membrane (OM)

A modification of the method of Filip *et al.*,[25] for the preparation of OM from *E. coli*, was used. Confluent growth from each of five DSA plates was washed off with 5 ml of 0·01 M HEPES buffer, pH 7·4. The bacteria were deposited by centrifugation (20 000 *g*, 15 min), resuspended in 20 ml of buffer, and disrupted in a French pressure cell (Aminco, Silver Springs, MD, USA) at 6000 psi. After removal of unbroken cells by centrifugation (10 000 *g*, 15 min), *c.* 400 μl of Sarkosyl (sodium N-lauroyl sarcosinate; Sigma; 30% w/v) was added to give a final concentration of 0·7%. The OM was deposited by centrifugation at 50 000 *g* for 1 h, washed in distilled water, resuspended in 1 ml of distilled water, and stored at −20°C.

SDS-PAGE and immunoblotting

OM proteins were analysed on slab gels 10% with Laemmli buffers,[26] and stained with Coomassie Blue.

The method of Hancock and Poxton[27] was used for immunoblotting. Separated proteins were transferred to nitrocellulose membranes (Sartorius, 0·2 μm pore-size), allowing transfer to proceed for 16 h at 12 V. For immunodetection by the BioRad system, the mouse sera were diluted 1 in 100, and the rabbit anti-mouse IgG-horseradish peroxidase conjugate (ICN Immunochemicals) was diluted 1 in 500.

Results

SDS-PAGE of outer membrane protein (OMP)

Fig. 1 shows the Sarkosyl OM preparations from 30 strains of *P. multocida* type A and Taxon 13, analysed by SDS-PAGE, and stained with Coomassie Blue. Overall, the patterns are very similar, revealing two, three or four major proteins (labelled *a, b, c* and *d*) with molecular masses (M_r) in the range 36·5–19 Kda, though each protein varied in M_r over a limited range. By visual examination, the strains could be arranged in 12 groups based on the mobility of these major OMPs (table I).

Proteins *b* and *d* were the most strongly stained and were present in all strains. The M_r of protein *b* was in the range 34·5–29·5 Kda. Protein *c* showed the most constant M_r—26 Kda in the majority of strains. Protein *a* was absent or incompletely

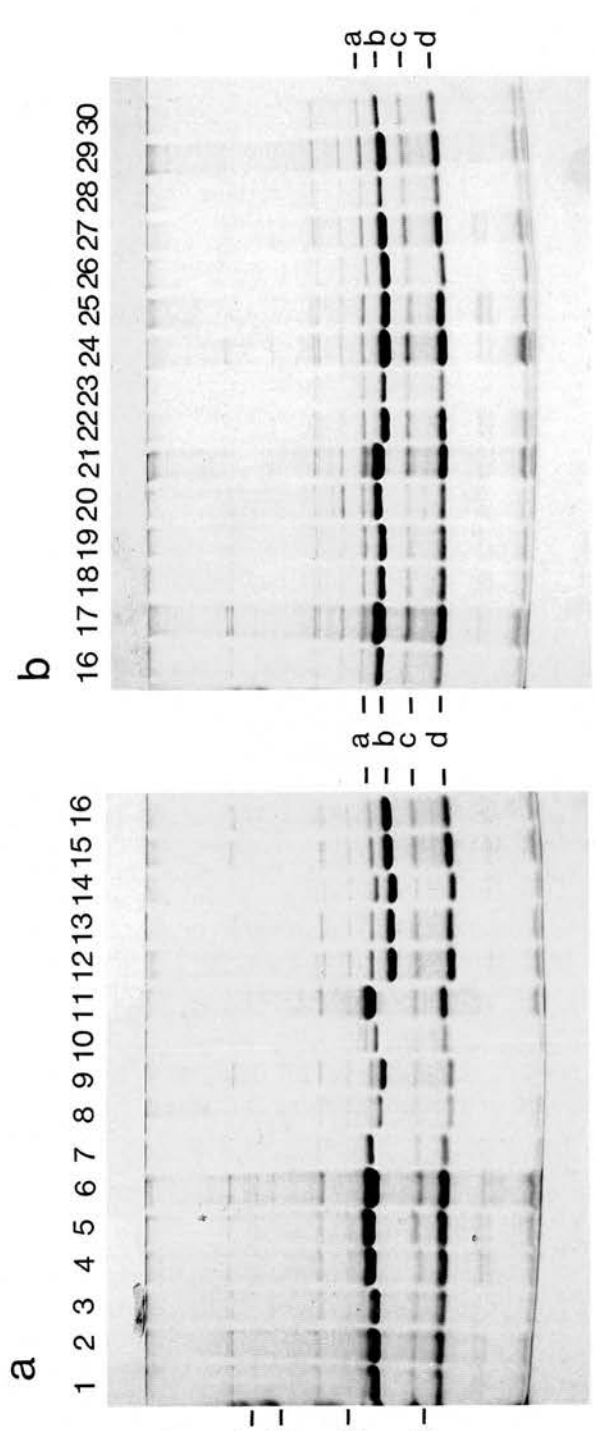

Fig. 1. SDS-PAGE gels of Sarkosyl OM preparations from strains of *P. multocida* and Taxon 13,* stained with Coomassie Blue; a, b, c, d represent major OMPs. **(a)** Track 1, strain X200; 2, W829; 3, X1113; 4, W571; 5, X198; 6, X1016; 7, X195; 8, W599; 9, W674; 10, X1043*; 11, NCTC 10322; 12, W819*; 13, X969; 14, W590*; 15, W833; 16, X109. **(b)** Track 16, strain X109; 17, X12; 18, X9; 19, W828; 20, X699; 21, X1122; 22, X918; 23, X1053; 24, X110; 25, X120*; 26, W577*; 27, X483; 28, W971; 29, W820*; 30, X1049.

Table I. Grouping of 24 strains of *P. multocida* and six strains of Taxon 13 with patterns produced by major OMPs on SDS-PAGE as shown in fig. 1

Group	Tracks (fig. 1)	Strains of *P. multocida* or Taxon 13
1	1 2 3	X200 W829 X1113
2	4 6 7	W571 X1016 X195
3	8 9	W599 W674
4	10	X1043*
5	5 11	X198 NCTC10322
6	12 13	W819* X969
7	14	W590*
8	15 16 17 18 19	W833 X109 X12 X9 W828
9	20 21	W669 X1122
10	22 23 24 25	X918 X1053 X110 X120*
11	26	W577*
12	27 28 29 30	X483 X971 W820* W1049

* Six strains of Taxon 13.

resolved in strains X200, W829, X1113, W571, X198, X1016, X195 and NCTC 10322; where present, its M_r ranged from 36·5 to 33·5 Kda. The M_r of protein *d* ranged from 21 to 19 Kda.

There were no features in the OMP patterns which allowed differentiation between the typical *P. multocida* and the Taxon 13 strains.

Mouse protection

Groups of mice were vaccinated with heat-killed cells of strains W674 and W829 (*P. multocida*) and strain X120 (Taxon 13). Vaccinated mice and non-vaccinated controls were challenged with 10 LD50 of the homologous strain and 16 heterologous strains representing most of the groups defined by SDS-PAGE of the OMPs (table II). Strain W674 appeared to afford the best protection—at least 50% protection against nine of the 17 challenge strains. Strain W829 gave this degree of protection against four strains, but the Taxon 13 strain X120 protected only against strain X9. Strain X120 was also the only strain that did not afford homologous protection at this level. There was no apparent correlation between the SDS-PAGE group pattern and protection.

Demonstration of antibody response to the major OMPs in vaccinated mice by immunoblotting

For each of the three groups of vaccinated mice, seven strains (against some of which vaccination was protective and some non-protective) were selected for investigation by immunoblotting with pre-vaccination and pre-challenge sera. With the pre-vaccination sera, immunoblots showed no reaction. In the pre-challenge sera, however, the mice had responded to at least two of the major OMPs; but there was no correlation between immunoblot pattern and protection. Proteins *a* and *d* were the most immunogenic. Immunoblots with pre-challenge sera from mice vaccinated with strain W829 are shown in fig. 2b. There was a relatively uniform response to both *a* and *d* proteins; but this did not depend upon the M_r being the same as that of the vaccine strain. It appears that there are common epitopes on all the *a* proteins and similarly on all the *d* proteins. The response to the other two major proteins was much more variable and appeared to be PAGE-group specific, e.g., strains W829 and X1113 in tracks 1 and 3 of fig. 2 both belong to PAGE group 1 (table I). There was, however, no correlation between the patterns and protection: vaccine W829 protected against the strains in tracks 1, 2, 3 and 6, but not against the strains in tracks 4, 5 and 7, although the immunoblots in tracks 4 and 6 are indistinguishable.

Discussion

The patterns produced on SDS-PAGE by Sarkosyl extracts of *P. multocida* are similar to the patterns produced by the OMPs of other gram-negative bacteria, in which there are a small number of major proteins and several minor ones.[28] The four major proteins which we have labelled *a–d* have allowed us to arrange strains of *P. multocida* into groups (table I). It is apparent from this grouping that strains of Taxon 13 share profiles of major OMPs with strains of *P. multocida*, and we suggest that these taxa cannot be distinguished on this basis.

Table II. Percentage protection of mice in groups of 10 vaccinated with heat-killed organisms and challenged with strains of *P. multocida* or Taxon 13

Vaccine strain	Percentage protection* against challenge with																
	P. multocida strain													Taxon 13 strain			
	W674 (3)†	W829 (1)	W669 (9)	W571 (2)	X9 (8)	X12 (8)	X110 (10)	X198 (5)	X971 (12)	X1016 (2)	X1053 (10)	X1113 (1)	X1122 (9)	X120 (10)	W577 (11)	W590 (7)	W819 (6)
W674	70	50	50	0	20	60	60	20	20	0	50	60	80	70	0	20	20
W829	50	90	0	0	60	0	0	...	10	0	30	50	40	0	0	0	20
X120	40	30	0	0	60	20	20	...	10	0	20	0	40	20	0	20	20

* Percentage protection $= \frac{\text{number of vaccinated survivors} - \text{number of non-vaccinated survivors}}{10} \times 100$

† In parenthesis: SDS-PAGE group no.—see table I.

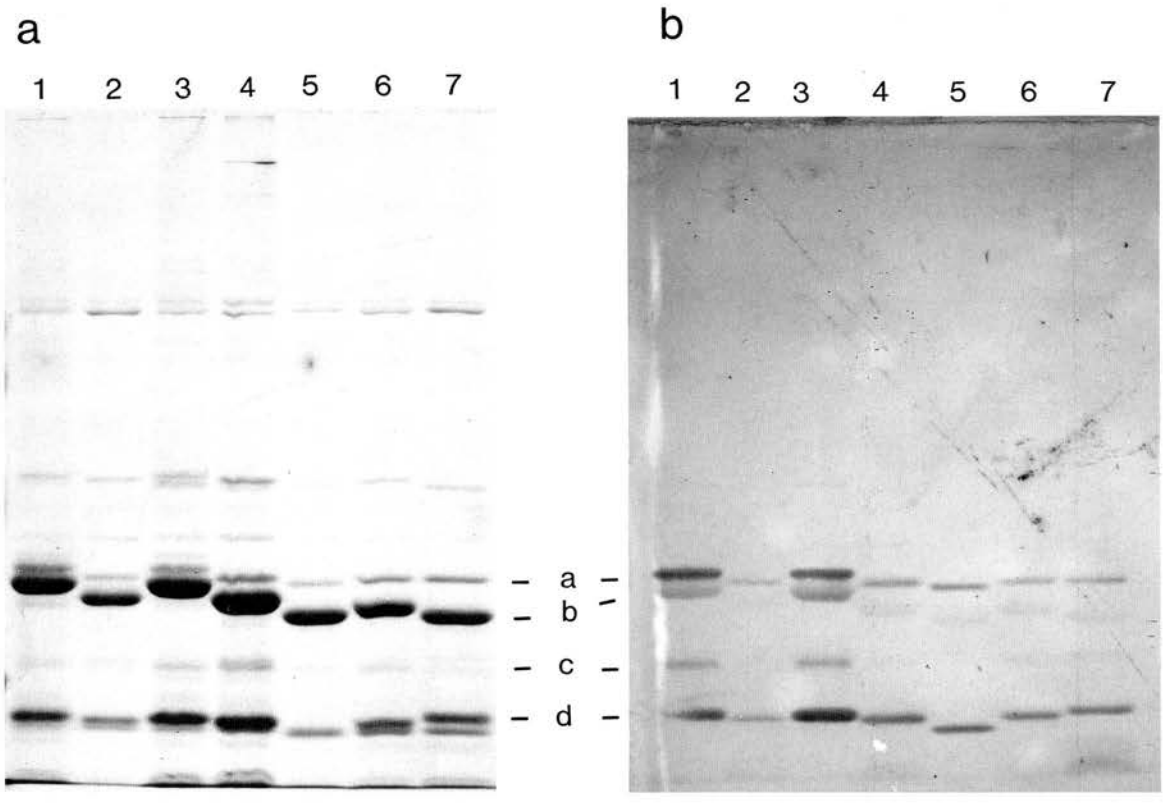

Fig. 2. (**a**) SDS-PAGE gels of Sarkosyl OM preparations from strains of *P. multocida* and Taxon 13* against which mice were or were not protected by vaccination with strain W829 (see table II). Track 1, strain W829 (protected); 2, W674 (protected); 3, X1113 (protected); 4, X12 (not protected); 5, W577* (not protected); 6, X9 (protected); 7, X120* (not protected). (**b**) Corresponding immunoblot probed with pre-challenge serum from mice vaccinated with heat-killed cells of strain W829. (a, b, c, d represent major OMPs).

When mice were vaccinated with heat-killed cells they produced an antibody response to two or more of the major OMPs (fig. 2b). This response was a combination of species-specific and PAGE-group-specific antibodies, but the latter did not correlate with protection. The proteins were antigenic in mice but not protective. Moreover, although proteins may differ from one another in M_r, they did not necessarily differ antigenically. This finding applies also to other groups of closely related species, e.g., some of the major OMPs of Enterobacteriaceae share epitopes but differ in M_r.[29]

Our conclusion is that, in this mouse model of pasteurellosis, the major OMPs are not protective antigens, and therefore may be unlikely candidates for vaccines. However, this appears to contradict the findings of Lu *et al.*[17] who showed that the 37·5 Kda OMP (presumably our protein *a*) was protective against homologous challenge in a rabbit model; but in a mouse model, protection was afforded against heterologous challenge only if the challenge strain was shown, by probing with a monoclonal antibody, to express the 37·5-Kda antigen.[30] Protection was not provided against strains lacking this antigen. Thus, they showed that this OMP can be a protective immunogen in the strains that possess it; but they concede that other antigens may be more important as vaccine candidates, because only 24% of their strains of *P. multocida* expressed this antigen.

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Production of mouse monoclonal antibodies to *Pasteurella multocida* type A and the immunological properties of a protective anti-lipopolysaccharide antibody

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Summary. Eight monoclonal antibodies (MAbs) were produced from mice immunised with whole cells of heat-killed *Pasteurella multocida* type A which had been cultured under iron-restricted conditions. The MAbs were selected by an enzyme-linked immunosorbent assay (ELISA) in which the antigen consisted of whole bacteria of the immunising strain. Their reactivity was investigated further by immunoblotting, indirect haemagglutination, a complement-mediated bactericidal assay and passive protection of mice. One of the eight MAbs was shown by immunoblotting to react with lipopolysaccharide (LPS), was bactericidal, and completely protected mice against homologous challenge with 10 LD50 of live bacteria. This MAb was selected for further study. Its reaction with LPS of 17 type-A strains and of single strains of types B, D and E was investigated by immunoblotting. Strains that reacted with the anti-LPS MAb in immunoblots were susceptible to its bactericidal activity and gave high ELISA absorbances. Those that did not react were not susceptible to its bactericidal activity and gave low ELISA readings. The relation between bactericidal activity and ELISA absorbance was highly significant ($p < 0{\cdot}001$). Five of the strongly reacting heterologous strains and one non-reacting strain were selected as challenge organisms in a passive protection experiment: only the mice receiving the reacting strains were protected.

Introduction

Pasteurella multocida is the cause of various diseases in mammalian and avian species.[1] Capsular type-A strains cause fowl cholera, pneumonia in cattle, sheep and pigs, and "snuffles" in rabbits;[1,2] strains of types B and E cause haemorrhagic septicaemia in cattle and buffaloes;[3] and type-D strains cause pneumonia in cattle and atrophic rhinitis in pigs.[1,4]

Capsular polysaccharides of *P. multocida* types B and E are protective against haemorrhagic septicaemia.[5] However, non-capsulate and capsulate organisms are equally effective in immunising birds against fowl cholera.[6] Lipopolysaccharide (LPS) of *P. multocida*, which is considered to be responsible for the somatic serotype specificity,[7] was poorly immunogenic in mice in its purified form, but protective in chickens.[8] A protein-LPS complex protected mice, rabbits and chickens.[9,10] LPS has been recognised as the major immunogen in ribosomal vaccines.[11,12] The role of outer membrane proteins (OMP) in protection against *P. multocida* infections is unclear. In mice, protection by whole-cell type-A vaccines was unrelated to their content of OMP;[13] in rabbits, however, a 37·5-KDa OMP has been identified as a protective antigen, but only against the strains that produce it.[14,15]

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Materials and methods

Bacterial strains

P. multocida strain W674, a calf pneumonia isolate, was used to immunise mice for MAb production. The National Collection of Type Cultures supplied strains of capsular serotypes A (NCTC 10322), B (NCTC 10323), D (NCTC 10325) and E (NCTC 10326). All other strains

were of *P. multocida* type A, isolated from calves at Veterinary Investigation Centres and submitted to the Moredun Institute for identification as described previously.[16]

Immunisation procedure and production of monoclonal antibodies

Five 6-week-old BALB/c mice were immunised with 10^8 heat-killed (60°C, 90 min) cells of strain W674, grown in nutrient broth (NB; Oxoid) containing 2,2′-dipyridyl; the iron-restricted medium probably increased the resemblance of the bacteria to those grown *in vivo*. A suspension of washed bacteria ($8{\cdot}0 \times 10^9$/ml in PBS) was emulsified with an equal volume of Bayol/Arlacel adjuvant. One volume of the emulsion was mixed with 3 volumes of a 1 in 10 dilution of alhydrogel. Each mouse received 0·1 ml of this mixture intraperitoneally (i.p.) on days 0 and 14. Blood was taken from the tail vein of each mouse on day 22 and the antibody response was measured by ELISA (see below) with an antigen consisting of whole cells grown in the presence of 2,2′ dipyridyl. The mouse with the highest antibody titre was selected for fusion. Three days before fusion (day 30+) the mouse was inoculated intravenously with heat-killed *P. multocida* (10^8 cells) in 0·1 ml of saline. Fusion with NS-0 cells was made by the method of Kohler and Milstein[17] except that polyethylene glycol was used as the fusion agent. Hybridomas producing specific antibody were detected by a whole-cell ELISA (see below) and were cloned and sub-cloned by limiting dilution.

Ascitic fluid was produced in adult BALB/c mice which had been primed i.p. with 0·5 ml of pristane (Sigma) 3 days before an injection by the same route of 10^7 hybridoma cells. Fluid was collected 7–10 days later.

Characterisation of MAbs

Isotype determination. The class and subclass of the MAbs in culture supernates were determined by means of a mouse monoclonal-antibody isotyping kit (RPN29: Amersham International plc) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA). An ELISA was used (a) to detect the antibody response of mice to vaccination, (b) to screen hybridomas for the production of specific antibody, and (c) to test the reactivity of MAbs against heterologous strains of bacteria. For purposes (a) and (b), *P. multocida* strain W674 was grown in NB containing 2,2′-dipyridyl for 6 h at 37°C, washed once in phosphate-buffered saline (PBS) and resuspended in carbonate-bicarbonate buffer, pH 9·6, to a concentration (10^7 cfu/ml) previously shown by chess-board titration to be optimal for coating. For (c), an overnight culture of each strain was grown in NB, washed once in PBS and resuspended in formol saline 0·5% to *c.* 10^7 cfu/ml (A420 = 0·1). The wells of the microtitration plates were coated with 100 μl of bacterial suspension by incubating overnight at 4°C. The primary antibody (100 μl) was either undiluted hybridoma culture supernate or doubling dilutions of mouse serum, after an initial 1 in 50 dilution, in PBS containing Tween 20 0·05% (PBST). After incubation for 1 h at 37°C and washing four times in PBST, 100-μl volumes of horseradish peroxidase-sheep anti-mouse Ig conjugate (Scottish Antibody Production Unit, Carluke) diluted 1 in 200 in PBST were added and incubated for 1 h at 37°C. After washing as previously, 100-μl volumes of substrate solution (ortho-phenylenediamine, Sigma; 0·4 mg/ml in citrate phosphate buffer, pH 5, with 30% hydrogen peroxide 4 μl/10 ml) were added and after 1 h at 37°C the colour was read at 492 nm in a Titertek Multiscan.

Immunoblotting. Whole-cell lysates of *P. multocida* strain W674 and Sarkosyl-extracted outer membranes prepared as described by Abdullahi *et al.*[13] were separated on polyacrylamide 10% gels with the buffer system of Laemmli.[18] LPS was prepared from several strains by the Proteinase K method of Hitchcock and Brown[19] and separated on polyacrylamide 14% gels with the same buffer system as that for the 10% gels except that sodium dodecyl sulphate was omitted from the separating and stacking gel buffers. Separated antigens were transferred to nitrocellulose membranes (Schliecher and Schuell; pore size 0·2 μm) and probed with undiluted hybridoma culture supernates for 4 h at room temperature. Immune complexes were detected with anti-mouse Ig-horseradish peroxidase conjugate and the BioRad HRP colour reagent.[20]

Indirect haemagglutination (IHA). This was performed essentially by the method of Carter.[21] Doubling dilutions of MAb (ascitic fluids), from 1 in 2 to 1 in 128, were tested against sheep red cells sensitised with capsular antigen prepared from the homologous strain W674 and several heterologous strains.

Bactericidal assay. MAbs were tested for their bactericidal capacity by the method of Wijewardana and Sutherland.[22] Briefly, triplicate samples (20 μl) of MAb (ascitic fluid) were incubated with 100 μl of a suspension of strain W674 (2000 cfu/ml) in modified barbitone buffer (Sigma) for 10 min at room temperature in microtitration plates (tissue culture grade; NUNC, Denmark). After the addition of 80 μl of undiluted fresh complement (serum from new-born calves or gnotobiotic lambs) to each well the plates were incubated for a further 30 min at 37°C. From each well, triplicate 10-μl samples were removed and plated on 7% sheep blood agar. The number of colonies (T_{30}) was counted after overnight incubation at 37°C. The initial bacterial inoculum was confirmed retrospectively by plate counts[23] and the assay count (T_0) was calculated as 0·5 × the mean cfu/ml. The percentage killing (K%) was then calculated by the formula:

$$K\% = 1 - \left[\frac{\text{mean cfu/ml at } T_{30}}{\text{mean cfu/ml at } T_0}\right] \times 100$$

Hyperimmune rabbit serum against strain W674 and fetal bovine serum were included as standard positive and negative sera respectively. The bactericidal capacity of the MAbs against heterologous strains was tested by the same method.

Passive protection of mice. The protective capacity of MAbs was investigated in BALB/c mice. Each MAb

(0·2 ml) in the form of ascitic fluid was inoculated into five mice. Ascitic fluid (0·2-ml volumes) of an irrelevant MAb (MAb E7, raised against louping-ill virus and kindly supplied by Mr M. Hussein of the Moredun Research Institute) was inoculated into five control mice. One hour later, all mice were challenged with approximately 10 LD50 of homologous or heterologous bacteria.[13] Mice were observed for 48 h after challenge, deaths were recorded and the percentage protected was calculated.

Results

Production and characterisation of monoclonal antibodies

Of 97 hybridoma supernates tested in ELISA, 20 gave optical densities (OD) at 492 nm of greater than 0·5. However, only eight continued to produce specific antibody after cloning and these were characterised further.

Isotype determination. Four MAbs (1/2.16.8, 1/15.9.1, 1/18.13.5 and 1/20.10.15) were of the IgM isotype, two (1/4.1.6 and 1/19.12.13) were IgG_1, one (1/13.6/12) was IgG_{2b} and one (1/8.16.11) was IgG_3.

Immunoblotting. By this technique, only two MAbs were shown to react with separated, transferred antigens of the immunising strain W674. When the outer membranes were probed, only MAb 1/4.1.6 reacted (with a 77-Kda band; fig. 1a). However, when LPS was used, another MAb, 1/8.16.11, reacted strongly with the material at the gel front, which corresponded to LPS. This is shown in fig. 1b. Included in this figure are tracks probed with pre-cloned and pre-subcloned supernates.

Indirect haemagglutination. Only one MAb, 1/2.16.8, demonstrated haemagglutinating activity (titre of 64) and was considered to be reactive with a component of the capsule. Subsequently, when this MAb was tested with heterologous strains, it was found to be specific for the immunising strain.

Bactericidal activity. Complement-mediated bactericidal activity was demonstrated only by the anti-LPS MAb (1/8.16.11). There was 80% killing of the homologous strain.

Passive protection of mice. The group of mice that received the anti-LPS MAb (1/8.16.11) resisted challenge with 10 LD50 of strain W674 and remained well during the 48-h observation period,

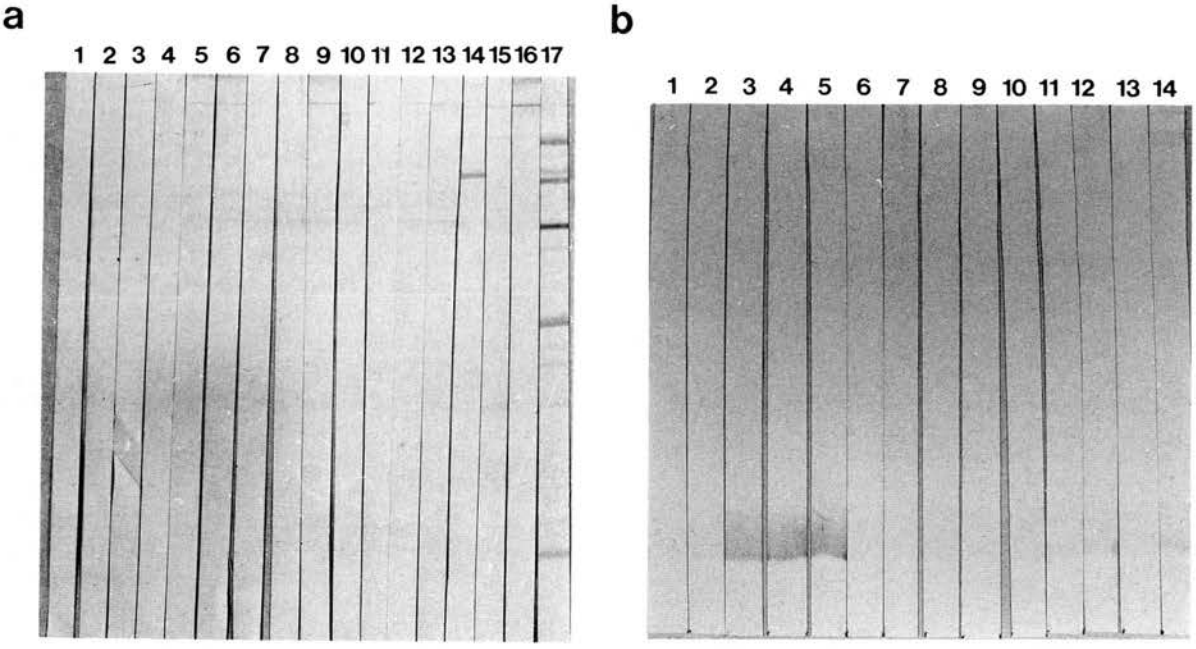

Fig. 1. Immunoblots of surface antigens of *P. multocida* strain W674 probed with undiluted hybridoma culture supernates. **(a)** Sarkosyl-prepared outer-membrane proteins: the only tracks showing positive reactions compared with the negative control (track 16, uninoculated culture medium) are 14 and 17, which correspond to MAb 1/4.1.6 and the positive control (serum from the mouse used for fusion) respectively. **(b)** LPS: the tracks showing strong, positive reactions were probed with MAb 1/8.16.11 (track 5), and supernates from the uncloned primary hybridoma culture well (track 3) and the first cloning of this culture (track 4); the serum from the mouse chosen for fusion is in track 14, and shows a weak positive reaction.

indicating 100% protection; all the control mice died. In the groups of mice inoculated with MAbs 1/19.12.13 and 1/20.10.15, two and five animals respectively were alive at the end of the observation period but they were severely ill. After killing the surviving mice by cervical dislocation, heart blood was plated on sheep blood agar. *P. multocida* was recovered from the sick mice but not from the mice protected with the anti-LPS MAb.

Reaction of anti-LPS MAb with heterologous strains

From the results described above, it was evident that the anti-LPS MAb (1/8.16.11) protected mice against homologous challenge and promoted complement-mediated killing. Therefore, this MAb was tested against heterologous strains in the whole-cell ELISA, in immunoblotting with LPS preparations, in bactericidal assays and by passive protection tests in mice.

The whole-cell ELISA results and the bactericidal capacities are shown in table I. The absorbances of heterologous strains are expressed as percentages of the OD_{492} of the homologous strain. Correlation between percentage killing and percentage absorbance, determined by the Spearman-Jackson rank correlation test, was highly significant ($p < 0.001$).

Immunoblots of LPS from 20 heterologous strains showed that 10 strains reacted strongly with the anti-LPS MAb and 10 did not (fig. 2). These reactions correlated with the ELISA and the bactericidal results. Fig. 3 shows that silver-stained polyacrylamide gels of the LPS preparations were all similar and resembled rough LPS of the enterobacteria.

The passive protection afforded by the anti-LPS MAb against six heterologous strains—five of which reacted in ELISA and in immunoblotting and one (strain A848) which did not—is shown in table II. The only strain against which no protection was obtained was A848; this strain was not killed by complement-mediated bactericidal activity. Protection was 60–100% against the other strains.

Table I. Bactericidal capacity of the anti-LPS MAb (1/8.16.11) against strains of *P. multocida* and the corresponding ELISA absorbances

Strain of *P. multocida*	Reactions of anti-LPS MAb with *P. multocida* strains	
	Bacterial killing (%)	ELISA absorbances (%)†
X1056*	100·0	91·6
A36*	100·0	97·0
A1174*	100·0	119·2
A757*	71·7	91·6
X120*	100·0	62·0
X110*	78·8	92·0
W674*	95·8	100·0
W666*	100·0	91·2
X1053*	80·0	95·7
A341*	100·0	102·6
Q110	12·0	11·5
Type A (10322)‡	0·0	14·3
Type B (10323)‡	5·0	14·9
Type D (10325)‡	0·0	12·4
Type E (10326)‡	0·0	8·2
A848	0·0	9·3
X1016	0·0	17·1
A26	0·0	10·4
A419	0·0	14·4
A586	0·0	16·0

*Strains that reacted with MAb in immunoblotting.
†ELISA absorbance values relative to the homologous (strain W674) reaction.
‡NCTC numbers.

Discussion

Of the eight MAbs that reacted in ELISA to whole bacteria, three were characterised further and shown by immunoblotting to be specific for LPS, capsule and an OMP respectively. The other five MAbs did not react in immunoblotting or IHA and were probably directed against protein epitopes which were irreversibly denatured during separation on polyacrylamide gel electrophoresis or on transfer to nitrocellulose.[24] As these MAbs were shown subsequently not to be protective in mice they were not investigated further.

The MAb that reacted only with the homologous capsular antigen in IHA did not mediate complement-dependent killing of bacteria *in vitro*. It did not protect mice against challenge with live organisms and was probably directed against a strain-specific surface antigen rather than to the type-specific hyaluronic acid capsule common to all type-A strains of *P. multocida*.

Similarly, the anti-77 Kda OMP MAb did not protect mice and was not bactericidal. Lu *et al.*[14] found that hyperimmune rabbit antiserum containing a high titre of IgG antibody to a 37·5-Kda OMP was protective in rabbits. However, they used a polyclonal serum containing antibody to several epitopes in a single molecule rather than a MAb defining a single epitope.

The most significant finding of the present study was that anti-LPS IgG_3 MAb protected mice against homologous and heterologous challenge by a mechanism associated with complement-depend-

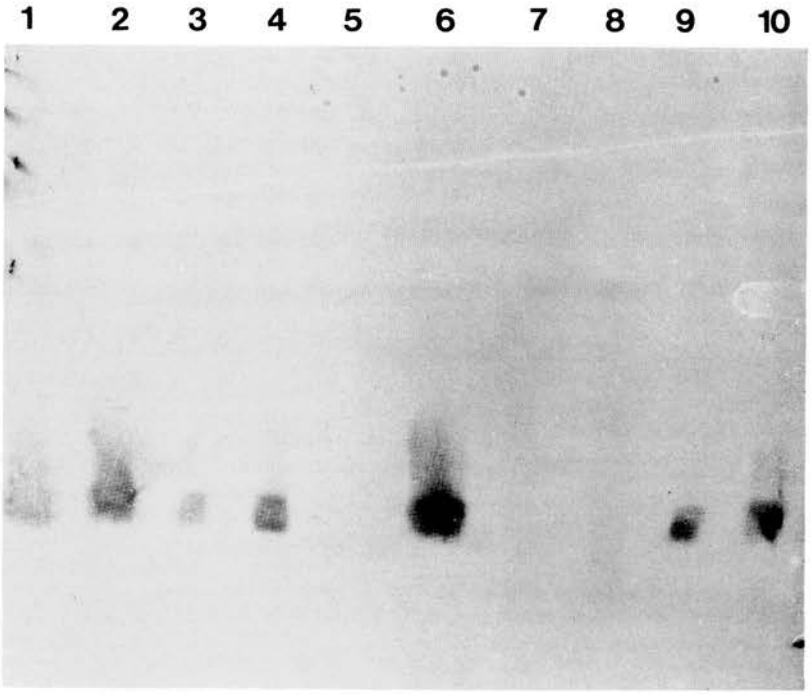

Fig. 2. Examples of immunoblots of LPS from homologous (track 9) and heterologous strains of *P. multocida* type A probed with anti-LPS MAb 1/8.16.11 diluted 1 in 2. Track 1, strain A757; 2, A36; 3, X110; 4, X1056; 5, A848; 6, W666; 7, Q110; 8, X1016; 9, W674; 10, X120.

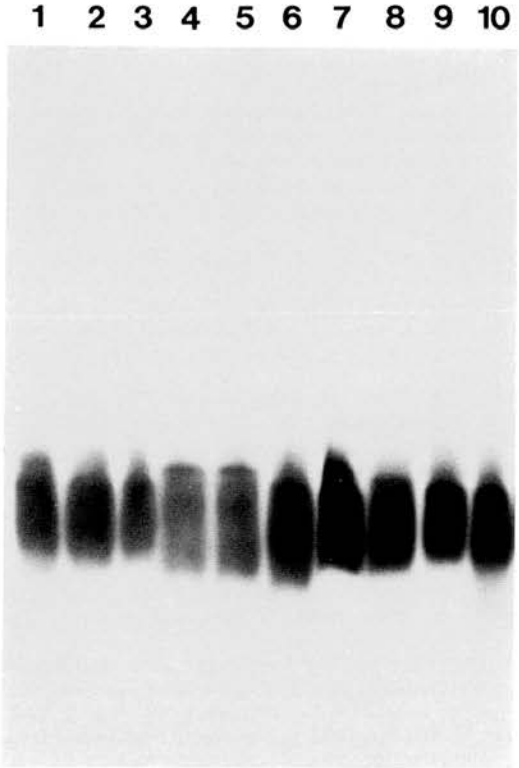

Fig. 3. Silver-stained LPS from 10 strains of *P. multocida* type A on polyacrylamide 14% gels. The order is as in fig. 2.

ent antibody-mediated bacterial killing. An investigation of the bactericidal activity of serum from vaccinated mice[22] suggested that a cell-surface antigen was associated with this mechanism of immunity, because the bactericidal capacity of mouse polyclonal serum was closely correlated ($p < 0{\cdot}001$) with whole-cell ELISA titres. It is clear that anti-LPS antibodies play a major role in immunity in mice by participating in complement-mediated bacteriolysis. This agrees with earlier views.[25,26]

In addition, the LPS of *P. multocida* type A has been identified as an important immunogen when

Table II. The activity of the anti-LPS MAb in passive protection of mice challenged with heterologous strains

Challenge strains		Number of survivors in groups of five mice
No.	LD50	
A36	$4{\cdot}8 \times 10^6$	3
X110	$3{\cdot}6 \times 10^5$	3
X1053	$8{\cdot}8 \times 10^6$	3
A341	$6{\cdot}3 \times 10^5$	5
A1174	$6{\cdot}1 \times 10^6$	4
A848	$1{\cdot}9 \times 10^3$	0

Mice were challenged with *c.* 10 LD50.

conjugated with protein in chickens, mice and rabbits.[8–10, 27–30] To induce immunity, ribosomal vaccines should contain LPS.[11,12] An LPS-ribosome complex gave an enhanced humoral but not cell-mediated immunity in chickens.[12]

The epitope recognised by the anti-LPS MAb was found to be present on the surface of about 50% of the capsule type-A strains investigated. It will be of interest to define the chemical structure of this epitope and to determine whether it is specific to one or more O serotypes in capsule type-A strains.

In any future work on vaccine development for *P. multocida* type A, consideration should be given to the presentation of the relevant epitope of the LPS in a non-toxic form—perhaps free of the lipid-A region—conjugated with protein. Anti-LPS MAbs that have complement-mediated bacteriolytic activity will be valuable tools in defining the epitopes of LPS that are important in stimulating protective immunity.

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The susceptibility of in vivo-grown *Pasteurella haemolytica* to ovine defence mechanisms in vitro

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Key words: *Pasteurella haemolytica*, in vivo; Immunity

1. SUMMARY

Pasteurella haemolytica organisms grown in vivo were examined for their susceptibility to ovine immune mechanisms in vitro. Compared with in vitro grown organisms they were less susceptible to opsonophagocytosis and, in contrast, susceptible to complement-dependent killing in the absence of exogenous antibody. These differences were not associated with phenotypic changes in the surface of the bacterial cell. However, overproduction and de novo synthesis of proteins was observed in in vivo grown organisms. Also, bound host-immunoglobulin was observed on in vivo grown organisms and a role for this in modifying the interaction with immune mechanisms is discussed.

2. INTRODUCTION

Pasteurella haemolytica is the causative organism of pneumonic pasteurellosis in sheep [1] and cattle [2]. There are two biotypes (A and T) of *P. haemolytica*, within which there are 16 serotypes. Biotype A serotype 2 is the commonest serotype isolated from field cases of pneumonic pasteurellosis in sheep [3,4].

Sera from lambs which have recovered from experimental infection with *P. haemolytica* A2 ("convalescent" sera) and from lambs vaccinated with serotype A2 antigens contain antibodies which can variously opsonise *P. haemolytica* A2 thus increasing its uptake by sheep bronchoalveolar macrophages (BAM) [5,6], activate complement-dependent bacterial killing [7], and neutralise the activity of the specific leukotoxin produced by *P. haemolytica* [6,8].

In vitro assays to measure these mechanisms of immunity correlated with resistance to infection [6–8] and immune sera passively protected recipient lambs against experimental infection [9]. Together, these findings suggest that the effector activities and titres of serum antibodies are important in immunity to *P. haemolytica* and that in vitro assays can be used to determine these criteria.

In vivo-grown bacteria can differ phenotypically from bacteria grown in vitro, differences including the in vivo expression of outer membrane proteins (OMPs) involved in iron acquisition [10–12] and changes in lipopolysaccharide (LPS) composition. Growth in vivo can also result in resistance to the bactericidal effects of serum. Sensitive *Neisseria gonorrhoeae* became resistant to human serum when grown in chambers implanted in the peritoneal cavity of guinea pigs, but

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reverted to the sensitive state on sub-culture in vitro [14].

The possibility that similar phenotypic changes may occur in *P. haemolytica* led to the studies reported here of the interaction of *P. haemolytica* grown in vivo in intraperitoneal chambers, with bactericidal and opsonophagocytic mechanisms.

3. MATERIALS and METHODS

3.1. Bacterial strains

Strain T884 of *P. haemolytica* A2, originally isolated from a field case of ovine pneumonic pasteurellosis, was used in all procedures.

3.2. Preparation, implantation and recovery of peritoneal growth chambers

Chambers for the in vivo growth of *P. haemolytica* A2 were prepared from silicone tubing (19.0 mm internal diameter and 25.4 mm outer diameter) (Altec, Alton, U.K.). Approximately 50 mm lengths of tubing were closed at one end by sealing in place a 25 mm diameter, 0.45 μm pore diameter filter membrane (Millipore, U.K.) using medical-grade silicone type A adhesive (Dow Corning, U.K.). The chambers, and a second filter membrane to be used subsequently to close the other end of the chamber, were sterilised by autoclaving.

Inocula for the chambers were prepared by centrifuging an 18 h culture of strain T884 in 10 ml nutrient broth (NB) at $10{,}000 \times g$ for 30 min, washing the pellet once in phosphate buffered saline (PBS) pH 7.2, and resuspending the bacteria in 50 ml of PBS. Approximately 10 ml vols were pipetted aseptically into the chambers, which were then sealed with a sterile filter membrane. Retrospective plate counting showed the inocula to contain 2.0×10^7 and 5×10^6 cfu ml^{-1} on two separate occasions.

Two chambers were each implanted aseptically into the peritoneal cavity of anaesthetised sheep. The peritoneal opening was closed with surgical suture and the animals allowed to recover. Seven days later the animals were killed and the chambers recovered.

The contents of the chambers (approximately 10 ml per chamber) were centrifuged at $10{,}000 \times g$ for 30 min at 4°C and pelleted bacteria washed once in PBS before resuspension in the required diluent (see below). The washed bacteria were tested for susceptibility to bactericidal activity and phagocytosis without further treatment. Aliquots of pelleted bacteria and supernatant fluid were also stored at −70°C or lyophilised for later analysis.

3.3. Susceptibility of in vivo-grown P. haemolytica A2 to phagocytosis

The susceptibility of in vivo-grown organisms to phagocytosis by BAM after opsonisation with either naive (standard negative) or "convalescent" (standard positive) SPF lamb serum was assessed by a plate-counting method. In vitro-grown bacteria were similarly tested as controls.

The washed in vivo-grown bacteria were resuspended in Hanks' balanced salt solution (Hanks') supplemented with 10% heat-inactivated (56°C for 30 min) foetal bovine serum (FBS, Gibco Paisley, U.K), 2% 30 mM Hepes buffer (Sigma), 1% sodium bicarbonate and adjusted to pH 7.2 with 1 M NaOH (Hanks' medium). In vitro-grown bacteria were prepared by inoculating 100 μl of strain T884 taken from storage at −70°C into 10 ml of nutrient broth (Oxoid, U.K.). The broth was incubated at 37°C for 18 h, then centrifuged at $10{,}000 \times g$ for 20 min. The pelleted bacteria were washed twice by centrifugation in Hanks' and finally resuspended in Hanks' medium.

Sheep BAM recovered from lungs obtained at a slaughter house by lung lavage [15] were sedimented at $100 \times g$ for 20 min. The cells were washed twice in Hanks' and resuspended in 5 ml of Hanks' medium. A 10 μl aliquot of cells was added to 90 μl of 0.4% trypan blue dye in PBS (Sigma, U.K.) and the total cell count and BAM viability were assessed using an improved Neubauer cell counting chamber. BAM suspensions were used only if $> 90\%$ were viable and if BAM represented $> 80\%$ of the cell population as assessed by morphological criteria. BAM were finally adjusted to 2.5×10^6 viable cells $\cdot$ ml^{-1} by dilution in Hanks' medium.

The opsonophagocytosis assay was performed on two separate occasions. Volumes (500 μl) of in

vivo- or in vitro-grown *P. haemolytica* A2 were mixed with 100 μl of the standard positive or negative serum in duplicate 1 ml polypropylene vials (NUNC, Denmark). Sera used for opsonisation were heat-inactivated at 56°C for 30 min before use. Tubes were incubated at 37°C for 30 min on a roller apparatus (Luckham Laboratories) to allow opsonisation to occur. Then, 400 μl of BAM suspension was added to each suspension and the tubes further incubated for 30 min on the roller apparatus to allow phagocytosis to occur. BAM were then sedimented by centrifugation at $100 \times g$ for 5 min at 4°C, washed three times in Hanks' to remove extracellular bacteria, resuspended in 1 ml of 0.1% Triton X100 (Sigma) and finally vortexed vigorously to lyse BAM and release intracellular bacteria. The lysed BAM suspensions were then titrated in ten-fold dilutions in peptone water (Gibco) and each dilution plated for bacterial counting.

The mean percentage of *P. haemolytica* A2 phagocytosed in duplicate sample tubes was calculated from the formula:

mean % phagocytosis = $100 \times$ (mean cfu $\cdot$ ml^{-1} in lysed BAM suspensions/$0.5 \times$ mean cfu $\cdot$ ml^{-1} in assay inoculum).

An opsonic index (OpI) [5] was calculated for each suspension using the formula:

$$\mathrm{OpI} = 100 \times (Cs - Cc/Ct),$$

Where Cs = mean count of phagocytosed bacteria in sample suspensions opsonised with the standard positive serum, Cc = mean count of phagocytosed bacteria in sample suspensions opsonised with the standard negative serum and $Ct = 0.5 \times$ mean count of total bacteria in the assay inoculum.

3.4. Assay of the susceptibility of in vivo-grown P. haemolytica A2 to bacterial killing mechanisms

In vivo-grown bacteria were tested on two separate occasions for their susceptibility to antibody-mediated, complement-dependent bacterial killing by the method described previously [7]. In vitro-grown organisms were tested in the same assay and considered as susceptible organisms [7]. "Convalescent" lamb serum and naive SPF lamb serum were used as the standard positive and negative sera.

The inocula for in vivo-grown organisms were prepared by resuspending organisms in modified barbitol buffer (MBB). Retrospective plate counting showed these to contain 1.7×10^4 and 1.1×10^4 cfu $\cdot$ ml^{-1} on the two separate assay occasions.

The in vitro-grown bacteria were prepared as described previously from 3 h cultures [7]. Retrospective plate counting showed these to contain 1.0×10^4 and 1.8×10^4 cfu $\cdot$ ml^{-1} on the two separate assay occasions.

3.5. Examination of proteins from in vivo-grown P. haemolytica A2 by SDS-PAGE

SDS-PAGE for the detection of proteins was carried out on 10% gels by the method of Laemmli [16]. Lyophilised whole bacterial cells (300 μg) from either peritoneal chambers or 18 h cultures in NB were resuspended in solubilising buffer and loaded onto 10% SDS-PAGE gels. Gels were stained with Coomassie blue dye [17] to visualise protein bands.

3.6. Detection of host-immunoglobulin bound to in vivo-grown organisms

Immunoglobulin bound to in vivo-grown *P. haemolytica* A2 was detected by Western blotting. Resolved proteins were transferred to nitrocellulose by the method of Burnette [18] as modified by Herring and Sharp [19] and reacted directly with donkey anti-sheep immunoglobulin conjugated with horse radish peroxidase (HRP, Scottish Antibody Production Unit, Law Hospital, Scotland).

3.7. Examination of LPS from in vivo-grown P. haemolytica A2 by SDS-PAGE

LPS from both in vivo- and in vitro-grown cells was examined by SDS-PAGE of proteinase K (Sigma) digested whole bacterial cells prepared by the method of Hancock and Poxton [20]. Lyophilised bacterial cells (300 μg) treated with proteinase K were loaded onto 12.5% gels. Gels were stained with silver stain [21] to visualise LPS.

3.8. Detection of bacterial capsule by Maneval stain

Detection of capsule was carried out retrospectively after the finding of phagocytosis studies. In vivo-grown organisms were therefore examined for capsule either after storage at −70 °C or upon recovery of organisms from a third chamber implant. Capsule was detected by the Maneval stain [22]. In vitro-grown *P. haemolytica* A2 cells were recovered from 10 ml vols of NB incubated at 37 °C for 6, 18 or 24 h. Bacteria were harvested from 1 ml vols by centrifugation at 11,000 × *g* for 5 min and pelleted bacteria resuspended in 1 ml of PBS, pH 7.2. Similarly, in vivo-grown bacteria were pelleted from chamber fluid by centrifugation followed by resuspension in 100 μl of PBS, pH 7.2. A loopful of each bacterial suspension was mixed on a microscope slide with a loopful of a 1.0% aqueous solution of Congo red stain (BDH Chemicals), spread thinly and air dried. The smears were then counter-stained with a 1.0% aqueous solution of acid fuchsin suspended in Maneval solution A (30 ml of 5% aqueous phenol, 8 ml of 20% aqueous glacial acetic acid and 4 ml of 30% aqueous ferric chloride) for 2 min, drained and blotted dry. By this method capsules appear as negatively-stained structures surrounding red staining bacterial bodies presented on a pale blue background.

4. RESULTS

At necropsy, implanted chambers were found to be completely enclosed by a thick fibrinous capsule (Fig. 1). Considerable areas of ecchymosis were noted in the peritoneum surrounding the chambers. White blood cells were not detected on any occasion in the chamber contents. This was taken as an indication that the chambers had remained intact. However, a small fibrinous clot was always present, and was removed, with sterile forceps. The pooled chamber contents contained 3.45×10^7 and 2.0×10^7 cfu · ml^{-1} *P. haemolytica* A2 in pure culture on two separate occasions.

The *P. haemolytica* inocula used for phagocytosis assays contained 2.9×10^7 and 1.0×10^7 cfu · ml^{-1} of in vivo-grown organisms and 3.9×10^7 and 1.0×10^7 cfu · ml^{-1} of in vitro-grown organisms respectively on the two separate assay occasions. Results of the phagocytosis assays (Table 1) showed that BAM phagocytosed a mean of

Fig. 1. Photograph of chamber implants in the peritoneum of a sheep. A fibrinous capsule from which the chambers were removed is arrowed.

Table 1
The mean percentage phagocytosis of *P. haemolytica* A2 grown in vivo or in vitro

Assay	Bacterial growth conditions	Opsonising serum	% phagocytosis	OpI
1	in vivo	positive [a]	9.65	7.8
		negative [b]	1.9	0
	in vitro	positive	21.8	21.8
		negative	0.002	0
2	in vivo	positive	8.1	3.4
		negative	4.7	0
	in vitro	positive	21.5	20.8
		negative	0.7	0

[a] Standard positive serum taken from a "convalescent" specific pathogen-free lamb.
[b] Standard negative serum taken from a naive specific pathogen-free lamb.
OpI–opsonic index.

21.8% and 21.5% of the in vitro-grown bacteria on two separate occasions in the presence of the standard positive serum and 0.002% and 0.7% in the presence of the standard negative serum. This percentage phagocytosis was similar to levels observed for *P. haemolytica* A2 previously [5,6]. The mean percentage phagocytosis of in vivo grown bacteria was also increased in the presence of the standard positive serum (9.65 and 8.1%) when compared to the standard negative serum (1.9 and 4.7%), but to a significantly lower degree ($P < 0.05$). The mean OpI for in vivo-grown *P. haemolytica* over the two assays was therefore 5.6%, compared with a mean OpI of 21.3% for in vitro grown bacteria.

These results indicated that in vivo-grown *P. haemolytica* A2 organisms were less susceptible to antibody-mediated opsonophagocytosis than in vitro-grown organisms and suggested that phenotypic changes might occur in organisms grown in vivo which reduce their susceptibility to opsonophagocytosis.

In the bactericidal assay 100% and 89% (means of triplicate samples) of in vitro-grown bacterial inocula were killed in the presence of the standard positive serum, on the two assay occasions, whereas no loss of viability (0% bactericidal activity) was detected with the standard negative serum. In contrast 97% and 100% of in vivo-grown bacteria were killed in the presence of the standard positive serum and 91% and 90% in the presence of the standard negative serum (Table 2).

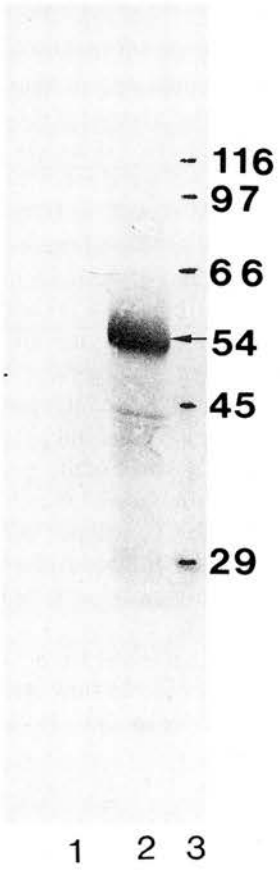

Fig. 2. Western blot of *P. haemolytica* A2 whole cells grown: in vitro (lane 1); in vivo (lane 2) and molecular mass markers (lane 3). Samples were reacted directly with donkey anti-sheep immunoglobulin conjugated with horseradish peroxidase to detect sheep immunoglobulin.

Table 2
The mean percentage killing (%K) of in vitro- and in vivo-grown *P. haemolytica* A2 in the bactericidal assay

Assay	Bacterial growth conditions	Serum	Mean % K
1	in vivo	positive [a]	97
		negative [b]	91
	in vitro	positive	100
		negative	0
2	in vivo	positive	100
		negative	90
	in vitro	positive	87
		negative	0

[a] Standard positive serum taken from a "convalescent" specific pathogen-free lamb.
[b] Standard negative serum taken from a naive specific pathogen-free lamb.
OpI–opsonic index.

These results suggested that either phenotypic changes in in vivo-grown organisms induced susceptibility to the alternative complement pathway or that host-derived immunoglobulin was bound to the cell surfaces of in vivo grown bacteria. On examination, host-derived immunoglobulin which was bound to in vivo-grown *P. haemolytica* A2 was detected on immunoblotting by direct interaction of the immunoglobulin with donkey anti-sheep immunoglobulin conjugated with HRP. A thick band of 53 kDa was detected by the conjugate on the track containing in vivo-grown

organisms. In vitro-grown organisms did not show this protein band (Fig. 2). Its molecular mass and reaction with the conjugate indicate this band to be the heavy chain of sheep IgG [6].

SDS-PAGE analysis showed extra protein bands and enhanced production of some other proteins in in vivo-grown cells compared to in vitro-grown cells (Fig. 3). These bands were 105, 100, 95, 70, 66, 53 and 23 kDa in molecular mass. Conversely, protein bands of 36 and 24 kDa were expressed in in vitro-grown organisms which were not detectable in in vivo-grown organisms. Examination of the SDS-PAGE patterns of proteinase K LPS preparations indicated that both in vivo- and in vitro-grown cells possessed rough type LPS (results not shown) which gave an identical profile to the serotype A2 LPS shown elsewhere [6]. No phenotypic change in the A2 LPS structure was therefore detectable after in vivo growth.

In vivo-grown bacteria were compared with early and late log phase in vitro-grown organisms for the presence of capsule. *P. haemolytica* A2

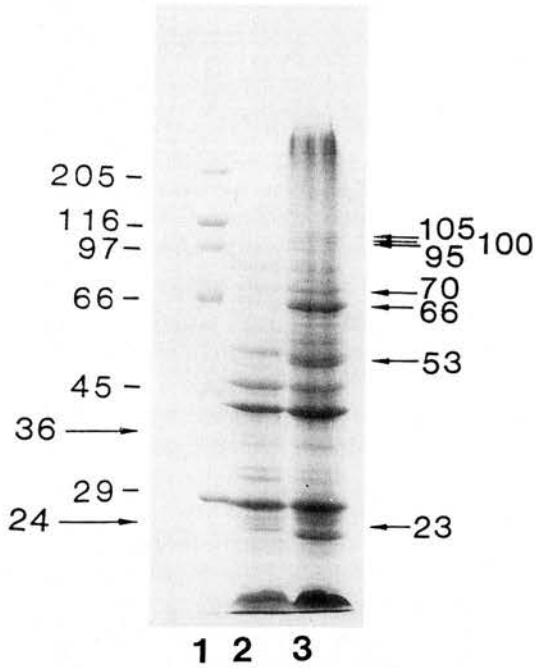

Fig. 3. SDS-PAGE of: molecular mass standards (lane 1); in vitro-grown (lane 2) and in vivo-grown (lane 3) *P. haemolytica* A2 whole cells.

organisms from 6 h in vitro cultures (Fig. 4A) were found to have visibly thicker capsules than bacteria from 18 and 24 h cultures (Fig. 4B and 4C, respectively). In vivo-grown organisms taken from storage at −70 °C (Fig. 4C) or taken directly from peritoneal chambers (not shown) had no more capsule than was seen on organisms from 18 h cultures. In vivo-grown organisms were, however, found to be aggregated into clusters of bacteria (Fig. 4D).

5. DISCUSSION

Intraperitoneal chamber-implants were successfully used in these studies to grow pure cultures of *P. haemolytica* A2 organisms in vivo. Relatively large numbers of viable bacteria free of contaminant host cells were recovered.

Day et al. [23] found that the formation of a fibrinous capsule around peritoneal implant chambers was associated with virulent strains of *Staphylococcus aureus* and considered that the phenomenon was probably due to chemotaxis, since the fibrinous capsule was composed mainly of neutrophils. In the present work, implanted chambers containing *P. haemolytica* A2 were also found to induce the formation of an enveloping capsule, suggesting that chemotactic factors are similarly produced by this bacterium. Areas of ecchymosis noted on the peritoneum adjacent to the chambers further suggest the production of soluble toxin(s) such as proteases [6,24] by the bacteria in the chambers.

The finding that in vitro-grown bacteria required the presence of both antibody and complement for bacterial killing to occur confirmed previous findings [7]. In vivo-grown organisms were as susceptible to antibody-mediated complement-dependent bacterial killing as in vitro-grown organisms, but were also killed in the absence of added specific antibodies. The detection of surface adherent (host-derived) antibodies on in vivo organisms may explain this finding. LPS has been identified previously as a target antigen for antibodies involved in bactericidal mechanisms against *P. haemolytica* A2 [7]. The finding that in vivo-grown organisms had no detectable structural

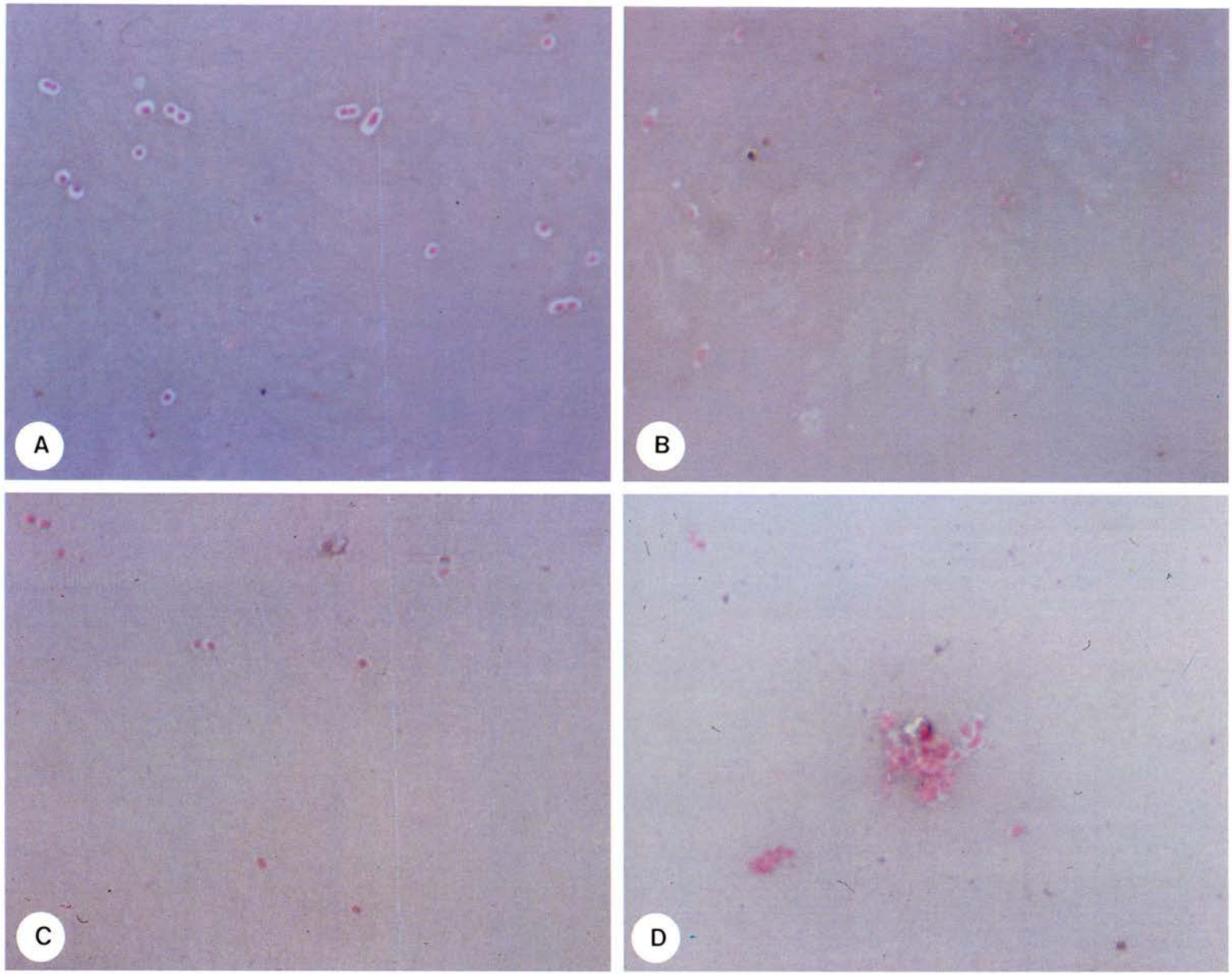

Fig. 4. Maneval stain showing the capsule surrounding *P. haemolytica* A2 organisms grown (**A**) for 6 h in vitro, (**B**) for 18 h in vitro, (**C**) for 24 h in vitro and (**D**) grown in vivo. Magnifications ×2000.

changes in LPS antigens compared with in vitro-grown organisms further supports the conclusion that host-bound immunoglobulin was responsible for the change in the susceptibility of in vivo organisms to bactericidal activity in the absence of exogenous antibody rather than this effect being due to phenotypic changes in LPS structure. The presence of surface-adherent, host-derived antibodies may also have been responsible for the slightly elevated levels of phagocytosis observed for in vivo grown organisms in the presence of the standard negative serum when compared to similar suspensions of in vitro-grown organisms. The binding of IgG to *S. epidermidis* grown in peritoneal dialysate has been observed by others [25].

The production of a capsule in *S. aureus* was associated with avoidance of opsonophagocytosis through the prevention of opsonin phagocyte-receptor interaction [26]. Early log-phase cultures of *P. haemolytica* A1 have been shown to produce more capsule than late log-phase cultures [27] and it is reported that early log-phase *P. haemolytica* A1 are less susceptible to opsonophagocytosis than late log-phase cultures [28]. The size of the *P. haemolytica* capsule has therefore been implicated as a factor in the avoidance of phagocytosis by this organism.

In vivo-grown organisms were opsonophagocytosed to a lesser degree than similarly treated in vitro organisms on two separate occasions. The thickness of the capsules produced by in vitro- and in vivo-grown organisms was therefore compared to determine if this factor might be implicated in the observed variations in susceptibility to opsonophagocytosis. In vivo-grown organisms were however found to possess similar amounts of capsule when compared to in vivo-grown organisms from 18 h cultures, which were employed for phagocytosis studies. Thus capsular size was not considered responsible for the observed reduction in susceptibility of in vivo-grown organisms to phagocytosis. In vivo-grown organisms were, however, found to be aggregated and this may have afforded some resistance to phagocytosis. Aggregation may indicate a change in cell-surface hydrophobicity which may be measureable in aggregation tests [29,30], or involve the interaction with host-derived factors such as fibrin, fibronectin, laminin and collagen which can cause clumping of other bacteria [31]. Alternatively, surface bound immunoglobulin may have caused aggregation through cross-linking. It is uncertain however, whether this aggregation would occur in natural infection or was an artefact of chamber growth.

The finding that early log-phase *P. haemolytica* A2 (6 h) cultures possessed larger amounts of capsule than organisms from 18 or 24 h cultures is in agreement with the observations on the A1 serotype [27].

Donachie and Gilmour [32] detected outer-membrane proteins in *P. haemolytica* A2 organisms obtained from the pleuritic fluid of experimentally infected lambs which were not detectable in in vitro-grown organisms. Similar expression of these antigens in organisms obtained from peritoneal chamber implants was therefore taken as evidence of phenotypic change occurring in the bacterial chamber inoculum in response to the in vivo environment. Organisms from chambers expressed increased amounts of 100 and 70 kDa antigens. These antigens were also detected by Donachie and Gilmour [32] in in vivo-derived organisms and were shown by them to be iron-regulated proteins (IRPs) that were inducible in vitro by growth in iron-depleted medium. In the present studies, two further proteins were detected in in vivo-grown organisms, these being 95 and 105 kDa in molecular mass. It is interesting to note that recombinant *P. haemolytica* cytotoxin has been found to consist of either a 102 kDa antigen [33] or of a 105 kDa antigen which reduces to a 95 kDa degradation product in older cultures [34]. The increased presence of 95 and 105 kDa proteins in the chamber-grown bacteria could therefore represent increased amounts of intracellular cytotoxin. Proteins of 53, 23 and 66 kDa were also detected as supernumerary in in vivo cells. The 53 kDa protein may have been heavy chain IgG, since bound IgG was detected on in vivo cells as a 53 kDa band. Purified ovine IgG has been shown to consist of a 53 kDa heavy chain and a 26 kDa light chain [6]. The 66 kDa protein was possibly sheep albumin bound to the in vivo cells.

Kelly et al. [13] have recently found that the LPS from in vivo grown *P. aeruginosa* organisms had additional polysaccharide bands which they

suggested enhanced the virulence of that organism. When examined for their LPS phenotype by SDS-PAGE, in vivo-grown *P. haemolytica* A2 organisms were found not to differ from in vitro-grown organisms.

No attempt was made to detect extracellular cytotoxin production by in vivo-grown organisms since substantial indirect evidence of toxin production in vivo has already been reported [6,35–38].

Two proteins (36 kDa and 24 kDa) produced by in vitro organisms were not expressed by in vivo organisms. These proteins may be useful in distinguishing between animals which have been vaccinated with in vitro-derived products and those which have undergone natural infection.

The findings that in vivo-grown organisms are susceptible to antibody-mediated bactericidal mechanisms and, to some extent opsonophagocytosis, further validate previous conclusions that these mechanisms play an important role in immunity against ovine pneumonic pasteurellosis (5 to 9) and that the in vitro assays for detecting these mechanisms may therefore help in analysing the protective potential of vaccines.

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THE CELL SURFACE ANTIGENS OF *PASTEURELLA MULTOCIDA*: DEFINITION OF A PROTECTIVE ANTIGEN IN TYPE A STRAINS.

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Keywords; *Pasteurella multocida*, monoclonal antibodies, lipopolysaccharide, protective antigen.

SUMMARY

The surface structure of *Pasteurella multocida* is typical of a Gram-negative bacterium and any of the component macromolecules might be considered to have the potential to confer protection. Two approaches have been used in an attempt to define protective antigens in Type A strains:

i) Whole, killed bacteria or cell surface fractions (outer-membrane, capsular extract and LPS) were used for active immunisation. Attempts were made to relate specific antibody levels to protection. A degree of protection was induced with every vaccine used, with heat-killed cells being marginally superior. As all vaccines, with the possible exception of LPS, contained more than one macromolecular species the specific protective antigen could not be defined directly. ELISA and immunoblotting were used to quantify the specific antibody levels to outer-membrane proteins and LPS. No correlation was observed between immunogenicity of outer-membrane proteins and protective capacity, while LPS appeared to be very poorly immunogenic and an antibody response could not be readily detected.

ii) Monoclonal antibodies (mAbs) were produced to defined cell surface components and used for passive immunisation. Of the mAbs selected several different specificities were identified. The only type to give a high degree of protection to homologous and heterologous challenge strains was shown by immunoblotting to bind LPS. It gave protection against only half the heterologous strains and this was directly related to LPS binding capacity. The LPS from the immunising strain has been fractionated and the protective epitope shown by inhibition of ELISA to be in the Lipid A region of the molecule.

We have concluded that LPS is a protective antigen but, at least in mice, is only weakly immunogenic.

INTRODUCTION

Pasteurella multocida is a small Gram-positive saccharolytic, facultatively anaerobic rod-shaped bacterium which is associated with several animal diseases of world-wide importance. Strains are conveniently classified into four capsular serotypes (1) and 16 O-serotypes (2). There is a partial relationship between capsular type and disease type: type A strains cause pneumonia in cattle, sheep and pigs, and "snuffles" in rabbits, strains of B and E types cause haemorrhagic septicaemia in cattle and buffaloes, and type D strains cause atrophic rhinitis in pigs and pneumonia in cattle (3). Vaccines are not available for all types, and those developed for protection against haemorrhagic septicaemia (HS) suffer from a protective capacity of short duration requiring re-vaccination at least yearly, an impractical proposal in many regions where HS is endemic.

When considering the candidate vaccine components for a Gram negative bacterium, molecules which might be included are capsular polysaccharides, lipopolysaccharide, outer membrane proteins, exotoxins and enzymes. The literature abounds with mention of all of these (4).

In the studies reported here, type A strains of *P. multocida* were chosen to investigate the identification of possible protective antigens in a mouse septicaemia model. Type A strains are characterised by possessing a capsule of hyaluronic acid, a poorly or non-immunogenic molecule (4).

The two approaches used involved: i) vaccinating with whole bacteria or semi-defined cell surface constituents and attempting to relate antibody levels produced against a certain component to protection, and ii) producing monoclonal antibodies (mAbs) to defined surface molecules and using these mAbs to immunise mice passively against challenge with live bacteria.

An important point to consider in such studies is an awareness that protective antigens might be only poorly expressed or not expressed at all on bacteria cultured in normal laboratory media. The expression *in vivo* might be significantly different from *in vitro*. Such factors as the concentration of iron are known to effect expression of both surface factors and toxins. Conditions in the host are usually extremely deficient in iron (5).

Much of the work presented in this paper has already been published as detailed papers (6, 7) and these should be consulted for methodology.

THE CELL SURFACE OF PASTEURELLA MULTOCIDA.

This small Gram-negative organism has the typical Gram-negative cell surface morphology: an inner and outer membrane are seen in the electron microscope, and a capsule is usually present. The classic mucoid/smooth/rough variation with the added phenomenon of iridescent or non-iridescent smooth types gives a range of phase variants. The basis of this phase variation is not totally understood but it is certainly related to virulence and capsulation.

As in many non-enterobacterial Gram-negatives the LPS is not the classical polymer with long, heterogeneous chains of O-polysaccharide, but is

a rough or perhaps semi-rough type and is probably more correctly termed a lipo-oligosaccharide or LOS (7, 8).

ACTIVE IMMUNISATION.

Various crude vaccines were prepared from 6 virulent Type A strains. Iridescent colonies were subcultured for 6h in Brain-Heart infusion broth, and vaccines produced were: whole, heat-killed cells (68°C for 90 min); formalin-killed cells (0.3% formalin in saline for 16h); "capsule" (a 2.5% NaCl extract, 56°C, 1h); and lipopolysaccharide (an aqueous-phenol extract). All were formulated with oil adjuvants: 5mg/ml antigen in water, mixed with an equal volume of Bayol F (Esso) containing Arlacel (Sigma) 10%. After two intraperitoneal (IP) injections (0.2 ml) 14 days apart, Swiss white mice were challenged IP with 10 LD50 of a 6 h broth culture 14 days after the last injection. Deaths were recorded for up to 48 h. Results showed that with homologous challenge it was possible to confer a degree of protection with all vaccine preparations (see table 1). The heat-killed (H-K) and formalin-killed (F-K) were however superior to the more defined preparations. It showed however, that components present in the LPS and "capsule" might be protective antigens.

In a further experiment the heat-killed vaccine was tested for cross-protection after challenge with heterologous strains (table 2). Results show that some strains conferred good cross-protection while others did not.

Table 1. Homologous protection after vaccination

Challenge strain	Survival* after vaccination with:				
	H-K	F-K	Capsule	LPS	No vaccine
W674	9/10	9/10	3/10	5/10	0/10
W829	10/10	10/10	8/10	7/10	4/10
Type A	10/10	9/10	4/10	5/10	5/10
X109	10/10	10/10	6/10	9/10	2/10
W828	7/10	3/10	2/10	2/10	1/10
W599	10/10	9/10	4/10	2/10	1/10

*Number surviving at 48 h/number challenged with 10 LD50

Table 2. Cross-protection by the heat-killed cell vaccine.

H-K vaccine	Percent protection after challenge with heterologous strain			
	W674	X109	W599	W829
W674	60	70	30	50
X109	10	40	30	10
W599	60	90	30	0
W829	60	40	10	90

SEROCONVERSION AND PROTECTION.

A major difficulty in defining protective antigens is that it is often difficult to prepare a pure antigen. Minor, but immunologically highly reactive contaminants can give a false impression. We attempted to get round this problem by assaying production of antibodies to specific antigens using immunoblotting (Western blotting).

Initial screening of seroconversion was performed by ELISA, but this technique, although sensitive, suffers from the same problem of knowing if the antigen on the plate is pure. Whole outer membranes were chosen as the initial screening antigen as this should contain outer-membrane proteins, LPS and possibly capsular material. The method of Filip et al (9), which uses the detergent Sarkosyl, was chosen to prepare outer membranes. When the outer-membranes were analysed by polyacrylamide gel electrophoresis (PAGE) and stained for proteins with Coomassie blue it was observed that four of the proteins (a, b, c, and d) were present in major amounts but of slightly varying molecular mass: strains could be grouped according to the pattern of these bands. Fig 1 shows PAGE protein patterns of 15 Type A strains. Of the 24 strains investigated so far, 12 different patterns have been recognised. In an attempt to check if a strain with the same outer-membrane pattern as another conferred cross-protection, immunoblotting, together with mouse protection was performed. Of the three strains selected the best protection was afforded by W674, which protected against 9 of the 17 challenge strains. The mice before vaccination had no antibodies detectable by immunoblotting to any outer-membrane antigen. After vaccination, all three produced a response to proteins a and d of all strains: they have shared antigenic determinants but are of varying molecular mass. The antibody response to

the other two bands appeared to be PAGE-group specific. An example of the Coomassie blue stained PAGE and the corresponding immunoblot is shown in Fig 2. There was no correlation between protection and the antigen pattern seen by immunoblotting.

By ELISA, when purified LPS was coated to plates, there appeared to be an anti-LPS response. However, despite being able to visualise LPS on gels by silver-stain (Fig 3), it was not possible to detect any reaction to LPS by immunoblotting. The conclusions from this part of the work were that it was possible to protect mice against a range of strains with a vaccine produced from a single strain, that the pattern of major outer-membrane proteins was not correlated with protection and that it was difficult to observe a specific anti-LPS response by immunoblotting.

MONOCLONAL ANTIBODIES AND PASSIVE PROTECTION.

Because of the difficulties encountered above, a second approach was to prepare monoclonal antibodies (mAbs) to cell surface components and attempt to use these to protect mice passively against live challenge. A heat-killed cell preparation of strain W674 was selected as the immunogen as this had been found to be the best at conferring wide protection in the active immunisation model. Bacteria were grown in an iron-restricted medium to mimic growth *in vivo*. After immunisation, the mouse with the highest antibody level (by ELISA) to whole iron-restricted bacteria was selected for fusion. Hybridomas were screened by whole cell ELISA, and eight were finally selected as stable clones producing antibodies which bound to whole cells.

CHARACTERISATION OF MONOCLONAL ANTIBODIES

By immunoblotting only two mAbs showed reaction, one binding to an outer-membrane protein and another to purified LPS. One further mAb

reacted in the indirect haemagglutination assay suggesting that it may be anti-capsule. In a complement-mediated bactericidal model only the anti-LPS antibody showed activity: there was 80% killing of the homologous strain.

In passive protection of mice, those animals receiving the anti-LPS mAb were 100% protected when challenged with 10 LD50 of the homologous strain, and remained well throughout. Two of the other mAbs, which had only reacted in the whole cell ELISA, showed some homologous protection, but the mice which remained alive at the end of the 48h observation period were severely ill.

THE ANTI-LPS MONOCLONAL ANTIBODY.

As this antibody was shown to protect passively against homologous challenge, it was studied for reaction with heterologous strains. In whole cell ELISA it reacted strongly with 10 of 20 strains tested. The LPS from the same 10 strains reacted in immunoblotting with the mAb, producing a band at the gel front (Fig. 4), and in the bactericidal assay the same 10 strains were effectively killed: between 72 and 100% killing compared to between 0 and 12% for the other strains. In the passive protection model the antibody protected between 60 and 100% of mice challenged with heterologous reacting strains but not against a non-reactor. We have so far been unable to perform "Heddleston typing" on our strains to see if reaction correlates with O-serotype.

In an attempt to determine the position of the protective epitope, the LPS was chemically fragmented and the fragments used in an ELISA inhibition reaction. The results are shown in Fig 5. The suggestion is that the lipid A portion is the site recognised by the antibody. This however

assumes that the LPS of *Pasteurella multocida* behaves in acetic acid in an analogous way to enterobacterial LPS. It might however be different, and the results simply suggest the epitope is acid labile. This requires further investigation.

CONCLUSIONS

Lipopolysaccharide, or lipo-oligosaccharide as it is more accurately described, is a protective antigen. In whole bacterial vaccines, or complex sub-cellular vaccines, it may be the lipopolysaccharide which is the important immunogen. In pure form however it is poorly immunogenic. The vaccine potential of this complex molecule should be investigated further, especially from the point of view of immunogenicity, adjuvanticity and T-cell dependency.

Although our experience has been restricted to type A strains in a mouse model, the approaches used could be applied to all types. It should be noted however that the capsule of Type A strains is hyaluronic acid which is not usually immunogenic. Types B and E are different in this respect.

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LEGENDS FOR FIGURES

Figure 1. SDS-PAGE of Sarkosyl-prepared outer membrane proteins from 15 strains of Type A *Pasteurella multocida* stained with Coomassie blue. a, b, c, and d are the major outer membrane proteins used for grouping the strains.

Figure 2. a) SDS-PAGE of Sarkosyl outer-membrane preparations from seven strains of *Pasteurella multocida* against which mice were protected (tracks 1, 2, 3, 6) or not protected (tracks 4, 5, 7) by vaccination with strain W829 (track 1). b) corresponding immunoblot probed with pooled pre-challenge serum from mice vaccinated with heat-killed cells of strain W829.

Figure 3. SDS-PAGE of lipopolysaccharide preparations from 16 strains of *Pasteurella multocida* stained with silver.

Figure 4. Immunoblots of LPS prepared from 10 strains of Type A *Pasteurella multocida* probed with the anti-LPS monoclonal antibody. Only 7 preparations gave reactions. This reflected the reactions in whole cell ELISA, the bactericidal model and in animal protection tests.

Figure 5. Inhibition of ELISA to show the location of the binding site of the anti-LPS monoclonal antibody. A cell envelope fraction was bound to the plates and the monoclonal antibody was preincubated with potential inhibitors. Lipid A, as well as the whole LPS and cell envelope fraction showed inhibition while the polysaccharide fraction of the LPS gave no inhibition.

Fig 1

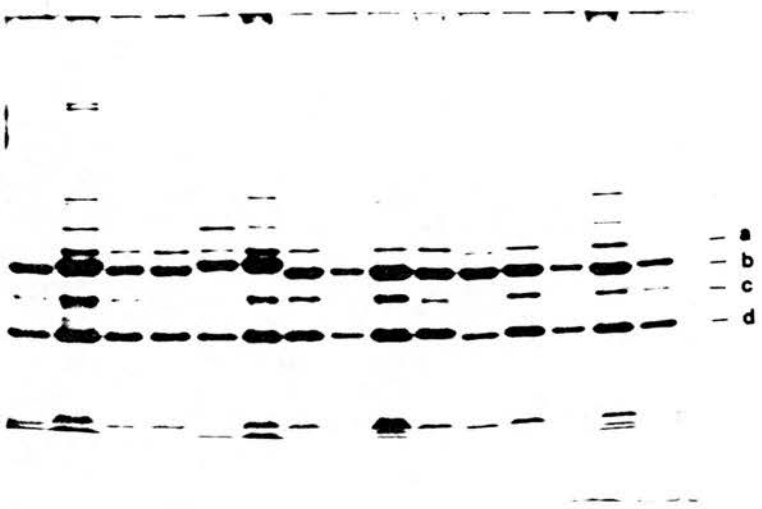


Fig 2

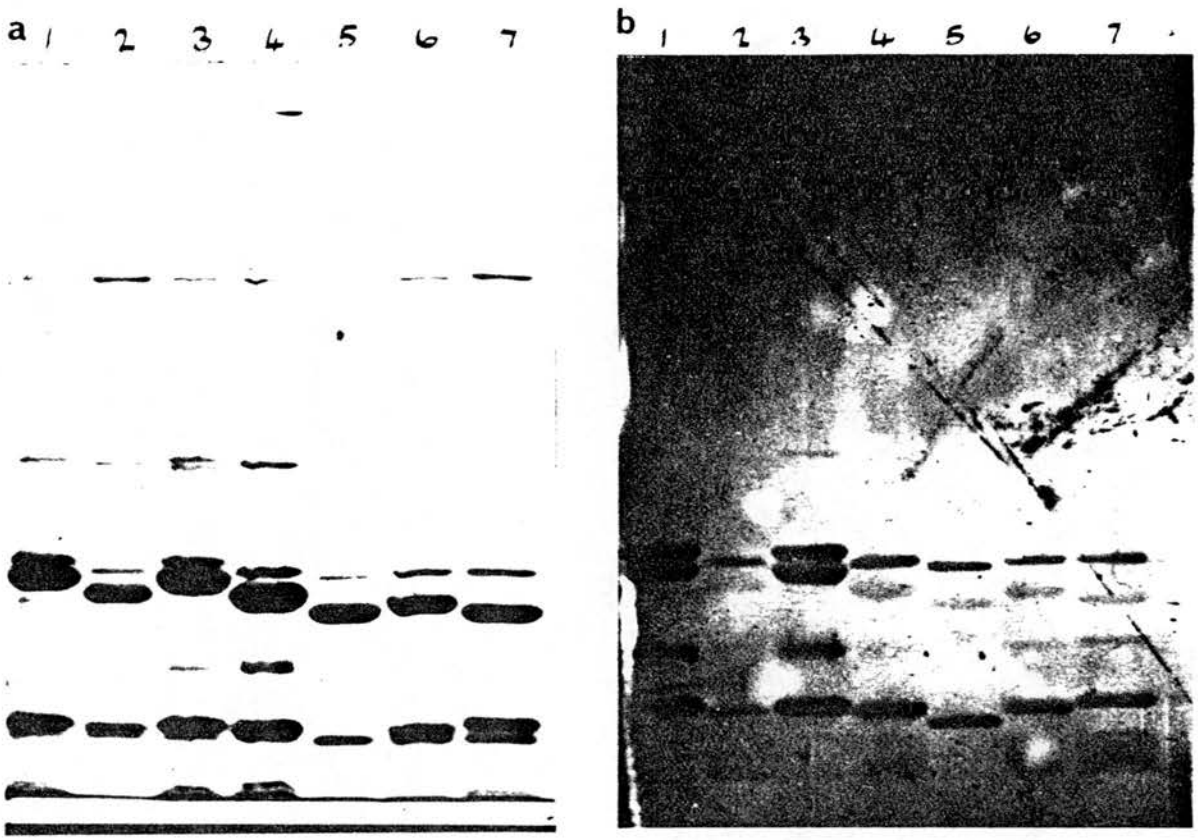


Fig 3

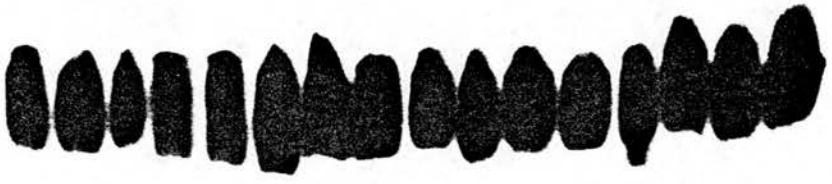
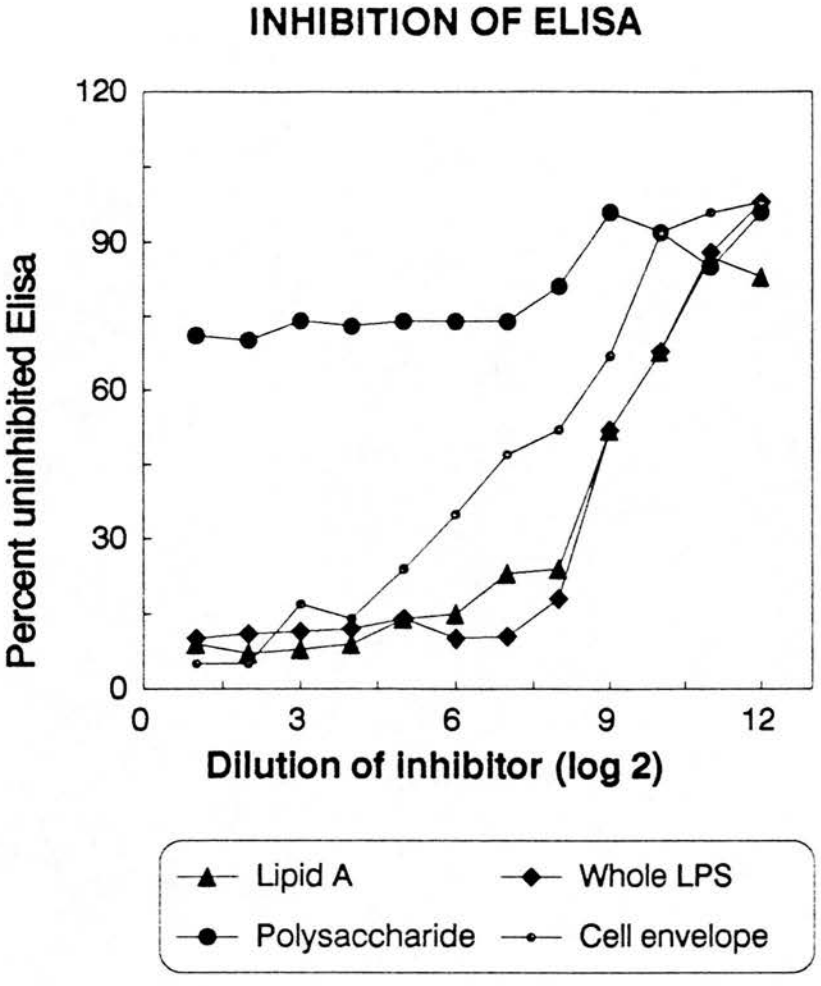


fig 4

Fig 5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Preliminary Study of the Anaerobic Bacteria Isolated from Subgingival Plaque from Sheep

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ABSTRACT

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A preliminary study was made to determine the genera of cultivable anaerobic bacteria which could be isolated from subgingival plaque of sheep. Samples were taken from 10 sheep on farms with a known record of broken mouth periodontitis. For assessment of the sampling technique, samples were also taken from freshly exposed tooth roots in killed sheep. The bacteria isolated on several selective and non-selective media were identified to genus level by a combination of Gram reaction, colony morphology and gas chromatographic analysis of volatile and non-volatile fatty acid metabolic end products. At least 10 different genera were isolated and these findings are discussed in relation to the bacteriology of human periodontitis and recent studies of sheep broken mouth periodontitis.

INTRODUCTION

Broken mouth periodontitis in sheep is an international economic problem for sheep farmers. The condition is in many ways similar to human periodontitis (Hatt et al., 1968; Cutress, 1976; Spence et al., 1980) with the formation of periodontal pockets and subsequent premature tooth loss. Histological studies of sheep have shown that subgingival plaque is associated with periodontal lesions (Cutress, 1976; Spence et al., 1980; Page and Schroeder, 1982), but little is known about the bacterial flora from these sites. Hatt et al. (1968) sampled with swabs plaque from broken-mouthed sheep isolating haemolytic and non-haemolytic streptococci, and fusiform bacilli together with small numbers of micrococci, *Neisseria* spp. and *Candida albicans*. However, no sampling or bacteriological details were given. In more recent studies (Frisken et al., 1986, 1987), black-pigmented *Bacteroides* spp. similar to *Bacteroides gin-*

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givalis have been found in increased levels in diseased sheep and a study (Frisken et al., 1988) was designed specifically to look in sheep for the suspected periodontopathic microorganisms commonly associated with human periodontitis. Black-pigmented *Bacteroides* spp., *Fusobacterium nucleatum* and *Fusobacterium necrophorum, Eikenella corrodens*, and *Capnocytophaga* spp. were isolated from sheep classified into groups according to severity of periodontal destruction.

This pilot investigation was aimed to determine the full range of genera of cultivable subgingival anaerobic bacteria that could be isolated from sheep at different stages of development of broken mouth periodontitis and to compare the information obtained with that known about the human condition. This should enable us to define the best sampling and cultural methods for more detailed study of sheep subgingival plaque.

MATERIALS AND METHODS

Sample collection

Two groups of five sheep were used, one from a farm with a bad record of broken mouths and the other from a farm with a low incidence of the disease. Before sampling, any food debris was removed with a gauze swab and the base of the tooth cleaned with a jet of air. Subgingival plaque samples were taken with sterile dental nerve broaches (Produits Dentaires S.A. Verey, Switzerland) from the disto-labial site on the central right incisor. The dental nerve broaches were pre-cut to fit the transport sample chamber described below. In addition samples were obtained at the Moredun Research Institute from four freshly killed sheep (killed for other purposes) by cutting a flap of gum away from the central incisor and scraping plaque directly from the labial surface of the tooth root, with sterile dental nerve broaches. One healthy site, one small pocketing and two deep pocketing sites were chosen and samples were taken from just below the gingival margin and from the base of the pocket. In addition, from the two deep pockets, a third sample was taken from between these two sites.

Sample transport and treatment

After sampling, the dental nerve broaches were immediately placed into the anaerobic conditions of the Anatube Type II transport system (Dept. Anaerobics, Microbiology, Ziekenhuis, Sint-Pieter, Brussels), In this system, a glass tube containing oxygen-removing catalyst pellets is fitted with a bored rubber stopper. A sample chamber is fitted into the underside of the stopper, and a plunger into the top side, which when depressed sends the sample chamber into the tube and coincidentally seals the tube. The sample chamber contains 0.2 ml of VMGII transport medium (shown to give good recovery rate of oral

B. melaninogenicus; Holbrook et al., 1978) containing 0.3% Ionagar, which is reduced by overnight incubation in an anaerobic cabinet (Forma Scientific, OH). The tubes are assembled and packed in anaerobic jars (B.T.L.) in the cabinet for transport to the farms.

On returning to the laboratory the tubes were immediately placed into the anaerobic cabinet, the samples recovered and diluted in pre-reduced volumes (1.8 ml) of nutrient broth. The samples were manually dispersed by repeated expression from pasteur pipettes. Volumes (20 μl) of 10-fold serial dilutions (from 10^1 to 10^4) were plated onto the pre-reduced culture media described below. All plates were incubated anaerobically in an atmosphere of 80% N_2, 10% H_2 and 10% CO_2 at 37°C for 5 days. From each plate, one colony of every morphological type was picked and plated out twice on CBA plates. One of the plates was incubated anaerobically as previously described for 2 days, while the second was incubated aerobically to rule out any organisms which might be facultative. Only the strictly anaerobic isolates were chosen for gas chromatographic (GC) analysis.

Culture media

Five agar isolation media were used, one non-selective: combined blood agar [CBA: columbia agar base (Oxoid) with horse blood, 4%; lysed horse blood, 1%; haemin, 5 μg ml^{-1}; menadione, 1 μg ml^{-1}] and four selective: (1) *Bacteroides*-selective agar (BSA; Holbrook et al., 1978) containing kanamycin (75 μg ml^{-1}) and vancomycin (2.5 μg ml^{-1}); (2) Rogosa *Veillonella* agar (RVA: Rogosa et al., 1958); (3) tryptic soy serum bacitracin vancomycin agar (TSBV; Slots, 1982), selective for *Actinobacillus actinomycetemcomitans*; (4) gentamicin combined blood agar (GCBA), as CBA with, in addition, 20 μg ml^{-1} gentamicin.

GC samples

Isolates were grown in PPYG medium (proteose–peptone, yeast extract medium supplemented with 1% glucose; Deacon et al., 1978) for 48 h, or for up to 4 days for slower growing organisms. After acidification to $\leqslant$pH 2 with 50% H_2SO_4, and centrifugation, the culture supernates were used directly for analysis of volatile fatty acids, or methylated at room temperature for 18–24 h by the method of Holdeman et al. (1977) for analysis of non-volatile fatty acids.

Samples (1 μl) were injected into 1.8-m$\times$4-mm glass columns packed with Supelco SP1220 (15%), H_3PO_4 (1%) on 100/120 mesh chromosorb WAW. The columns were heated isothermally at 147.5°C in a Pye Unicam Series 104 Chromatograph fitted with dual F.I.D., with oxygen-free nitrogen as the carrier gas, set at a rate of 35 ml min^{-1}. Temperatures for the injector and detector were 147.5 and 250°C, respectively, and the attenuation was 10^3. The recorder

speed was 300 mm h^{-1}. Before use, the columns were conditioned at 170 °C for 16–18 h with the carrier gas set at 20 ml min^{-1}.

Identification and approximate quantitation of the acids found in the test samples was achieved by comparing their retention times and peak heights with those obtained from standard mixtures of acids of known concentration. A concentration of < 0.2 mol l^{-1} acid was considered negative, 0.2–1.0 mol l^{-1} as trace, between 1.0 and 10 mol l^{-1} as a minor amount, and > 10 mol l^{-1} was considered a major amount (Deacon et al., 1978).

Identification of genera

Isolates were identified, as far as possible, to genus level using Gram-stain reaction, colony morphology and GC analysis of volatile and non-volatile fatty acid metabolic end products (Holdeman et al., 1977; Brown et al., 1989).

RESULTS AND DISCUSSION

The purpose of this study was to investigate the range of genera of cultivable anaerobic bacteria in sheep subgingival plaque and to compare this with the information known about human periodontal plaque. Sheep were chosen to cover different stages of disease development from relatively healthy mouths to ones near to tooth loss. As single samples only were taken, no attempt was made to assess clinically the condition of the sheep or to quantitate the bacterial genera. Investigations of plaque from various animals have demonstrated a flora similar to that of humans (Dent and Marsh, 1981; Laliberté and Mayrand, 1983). Samples were obtained from subgingival plaque taken from five sheep on each of two farms; one with a low incidence of broken mouth periodontitis and one with a bad record. On the five media used, the genera of anaerobic bacteria isolated are shown in Table 1. *Actinobacillus actinomycetemcomitans* was not recovered from any of the samples on the TSBV plates, and only one isolate of *Veillonella* species grew on RVA. The BSA grew a variety of different genera; however, the recovery of asaccharolytic black-pigmented *Bacteroides* spp. was found to be greater on the non-selective media. Indeed, van Winkelhoff and de Graaff (1983) found vancomycin at a concentration of 3.5 μg ml^{-1} (2.5 μg ml^{-1} was used here) to be inhibitory to *Bacteroides asaccharolyticus*. It was noticeable that every genus identified grew on GCBA except *Veillonella*; gentamicin was added to reduce aerobic growth and was particularly useful in two cases where the CBA, BSA and RVA plates were overgrown by an aerobic swarming Gram-negative bacillus (*Proteus* sp.).

The genera found in this study were *Bacteroides* (including black-pigmented spp.), *Fusobacterium, Lactobacillus, Clostridium, Peptostreptococcus*, other anaerobic cocci, *Actinomyces, Propionibacterium, Eubacterium, Bifidobacterium*

TABLE 1

Genera of anaerobic bacteria isolated from sheep subgingival plaque on different media

Genus	Growth on[1]				
	CBA	GCBA	BSA	RVA	TSBV
Bacteroides	+	+	+	+	+
Fusobacterium	+	+	+	+	+
Lactobacillus	+	+	+	–	+
Clostridium	+	+	+	–	–
Peptostreptococcus	+	+	–	–	–
Other anaerobic cocci	+	+	–	–	–
Actinomyces	+	+	+	–	–
Propionibacterium	+	+	–	–	–
Eubacterium	+	+	+	–	–
Bifidobacterium	–	+	–	–	–
Veillonella	–	–	–	+	–

[1]CBA, combined blood agar; GCBA, gentamicin combined blood agar; BSA, *Bacteroides*-selective agar; RVA, Rogosa *Veillonella* agar; TSBV, tryptic soy serum bacitracin vancomycin agar.

and *Veillonella*. Although spirochaetes were not cultured they were observed by dark-ground microscopy in every sample taken. These results compare well with recent human studies (Moore et al., 1982; Dzink et al., 1988) except that no *Eikenella, Selenomonas, Capnocytophaga* or *Wolinella* spp. were detected in the study reported here. Frisken et al. (1988), however, found *Eikenella corrodens*-like organisms and a few *Capnocytophaga* spp. in sheep plaque using media selective for these species.

The *Clostridium* spp. isolated are likely to have originated from food or soil contamination; it was noted that compacted food could be found at the base of the pockets. Tanner et al. (1979), however, describe the isolation of *Clostridium ramosum* from human periodontal sites.

Actinobacillus actinomycetemcomitans has been found in elevated numbers in active sites of localized juvenile human periodontitis (Slots and Genco, 1984) and was found to occur naturally in 69% of a population of squirrel monkeys (Clark et al., 1988). A selective medium for this organism was included in this study as it has been suggested that the clinical signs of broken mouth periodontitis resemble those of localized juvenile human periodontitis (Cutress and Ludwig, 1969). However, this organism was not detected here, nor in the studies of Dreyer et al. (1986) or Frisken et al. (1988).

To determine whether the sampling procedure used was removing a representative sample of the microbial flora of the subgingival plaque, samples were taken directly from the base of the pocket after excision of the gum in freshly killed sheep. This also enabled sampling from different parts of the pocket, to look at the distribution of genera along the tooth root surface. Single sites in

each of four different sheep were used, one healthy site and three with loss of attachment.

We found that the genera of organisms were constant in samples scraped from teeth at various depths of the periodontal pocket, and no differences were evident between sheep or between healthy and diseased sites. The number of samples taken was too small to make any conclusions from this, and the individual species involved need to be identified. The incidences of genera obtained from the samples taken from live sheep and from those scraped directly from exposed teeth are compared in Table 2. The genera and their incidences are comparable, indicating that similar plaque samples were obtained with the two sampling methods. There are also no obvious differences between the incidences of genera isolated on the two farms used in this study (Table 2).

Although this study was not quantitative, it was observed that most of the isolates belonged to the *Bacteroides* or *Fusobacterium* genera. Further identification of the *Fusobacterium* spp. (results not shown) revealed that the majority were *F. nucleatum*-like or *F. necrophorum*-like, as demonstrated by Frisken et al. (1988). However, few of the *Bacteroides* spp. fit into the recognized species except for the asaccharolytic black-pigmented spp., which were mostly *B. asaccharolyticus*-like or *B. gingivalis*-like (results not shown). Frisken et al. (1988) have shown increased incidence of *B. gingivalis*-like organisms in bro-

TABLE 2

Incidence of genera of anaerobic bacteria isolated from subgingival plaque samples obtained from periodontal pockets or samples obtained directly from tooth roots

Genus	No. of samples from which genera were isolated			
	Periodontal pocket samples[1]			Root sample[2]
	Farm A[3]	Farm B[4]	Total	
Bacteroides	3	5	8	10
Fusobacterium	4	5	9	10
Lactobacillus	3	3	6	4
Clostridium	2	4	6	4
Peptostreptococcus	1	1	2	2
Other anaerobic cocci	1	1	2	5
Actinomyces	1	1	2	3
Propionibacterium	2	3	5	5
Eubacterium	0	2	2	3
Bifidobacterium	1	2	3	4
Veillonella	1	0	1	0

[1]Subgingival plaque samples were taken from five sheep on each of two farms.
[2]Subgingival plaque samples were taken directly from freshly exposed tooth roots.
[3]Farm A: designated as having a low incidence of periodontal disease.
[4]Farm B: designated as having a high incidence of periodontal disease.

ken mouth periodontitis-associated sites compared with healthy ones, and Dreyer et al. (1986) found that antibody titres against the human isolates of *B. intermedius* and *B. gingivalis* were raised in diseased sheep in comparison with their flock controls. These observations indicate the possible involvement of the black-pigmented spp. in the pathogenesis of broken mouth periodontitis. In addition, in squirrel monkeys, the presence of black-pigmented *Bacteroides* spp. was associated with increased age, increased gingival index and the presence of calculus (Clark et al., 1988).

As all of the genera isolated in this study grew on GCBA (except *Veillonella* spp., of which only one colony was found) it is proposed that this medium is used in an extended study to examine the species of anaerobic bacteria associated with subgingival plaque during the development of broken mouth periodontitis in sheep.

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A longitudinal study of the cultivable subgingival anaerobic bacteria isolated from sheep during the development of broken mouth periodontitis

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Summary. In a longitudinal bacteriological study of the cultivable subgingival anaerobic flora isolated from developing broken mouth periodontitis in sheep, samples were taken from five sheep on each of three farms on seven occasions over a period of 2·5 years. Ten different bacterial genera were isolated regularly but with fluctuating frequencies. *Bacteroides* and *Fusobacterium* organisms accounted for nearly 70% of the isolates. The *Bacteroides* and *Fusobacterium* isolates studied in detail from one farm were identified to species level. The fusobacteria comprised *F. nucleatum*-like organisms (68·6%). *F. necrophorum* (29·6%) and *F. naviforme* (1·8%). The *Bacteroides* spp. were divided into 11 main groups and included black-pigmented species similar to *B. asaccharolyticus* and *B. gingivalis*. On the farm studied in detail, the sheep could be allocated to two groups according to progression of periodontal disease. Most of the *B. gingivalis*-like isolates were from sheep with actively progressing disease, indicating that this organism may play a role in periodontal destruction in sheep.

Introduction

Periodontal disease (broken mouth) in sheep is in many ways similar to human periodontitis.[1,2] It is a chronic inflammatory disease of the periodontium associated with alveolar bone loss which results in premature loss of the incisor teeth. The association of subgingival plaque with periodontal lesions has been shown histopathologically.[1–3]

Recently, several bacteriological studies in sheep have indicated the presence of anaerobic bacterial species similar to suspected periodontopathic organisms associated with periodontal disease in man.[4–6] However, a study of the complete cultivable anaerobic subgingival flora of sheep has not been made. As histopathological studies show that 2–3 years elapse between the first evidence of severe gingivitis and tooth loss,[7] a longitudinal study of the anaerobic flora isolated from subgingival plaque during this period may indicate the organisms that are important in periodontal disease of sheep.

The aim of this study was to perform such a longitudinal study of the subgingival flora of developing broken mouth periodontitis of sheep over a 2·5 year period and to relate the results to studies in man and other animals. A preliminary study, investigating the range of genera of anaerobic bacteria cultivable from the gingival crevices of healthy and diseased sheep[8] provided information on suitable sampling procedures and cultural requirements and these methods were employed in the longitudinal study.

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Materials and methods

Sample collection

From 15 flocks included in an epidemiological survey of broken mouth periodontitis in the South-east of Scotland, three flocks of Scottish Blackface sheep were selected. The flocks, on three separate farms, represented high, intermediate and low incidences of broken mouth periodontitis. Five ewes, each approximately 2·5 years old, were chosen at random from each flock at the first visit. Duplicate samples were collected by means of dental nerve broaches (Produits Dentaires S.A. Verey, Switzerland) from the same site (disto-labial site on the central right incisor) on three separate occasions throughout the year as previously described,[8] for a period of 2·5 years: the first sample was examined for cultivable bacteria, the other by dark ground microscopy.

Clinical examination

Each sheep was examined clinically and pocket depth, crown height, tooth movement, recent recession, gingivitis and occlusion were measured and scored as described by Spence and Aitchison.[9,10]

Sample treatment

After sampling, the dental nerve broaches were immediately placed into 0·2 ml of pre-reduced VMGII transport medium[11] under the anaerobic conditions of the Anatube Type II transport system.[8] On return to the laboratory, samples were recovered inside an anaerobic cabinet (Forma Scientific, OH, USA) and diluted in pre-reduced 1·8-ml volumes of nutrient broth. Samples were dispersed by repeated expression from pasteur pipettes (sheep plaque is not calcified). Volumes (20 μl) of five-fold serial dilutions from 10^1 to $1{\cdot}56 \times 10^5$ were plated on to pre-reduced Gentamicin Combined Blood Agar (GCBA: Columbia agar containing horse blood 4%, lysed blood 1%, gentamicin 20 μg/ml, haemin 5 μg/ml and menadione 1 μg/ml). This medium has been shown to support growth of the majority of anaerobic bacterial genera commonly isolated from sheep subgingival plaque.[8] Plates were incubated anaerobically in an atmosphere of N_2 80%, H_2 10%, CO_2 10% at 37°C for 5 days. For total anaerobic and aerobic counts, duplicate CBA plates (GCBA plates without gentamicin) were inoculated similarly and one set was incubated anaerobically as before, while the other set was incubated aerobically at 37°C for 2 days.

For an accurate analysis of the bacterial composition of the samples a predetermined number of colonies was selected, in an unbiased manner, to obtain a representative cross-section of the cultivable flora. From each sample a plate containing approximately 300 colonies was chosen and placed on a grid, consisting of 192 numbered hexagons. With computer-generated random numbers, 30 colonies were subcultured from the correspondingly numbered hexagons, and plated out twice on CBA plates. One set of plates was incubated anaerobically for 2 days, or up to 4 days for slower growing organisms, while the duplicate plates were incubated aerobically. Only strictly anaerobic isolates were investigated further by gas chromatographic (GC) analysis and, where selected, for identification to species level.

GC analysis

Sample preparation. Isolates were grown in PPYG medium (proteose peptone, yeast extract medium supplemented with glucose 1%[12]) for 2 days, or up to 4 days for slower growing organisms. After acidification to pH 2 or less with H_2SO_4 50% (v/v) and centrifugation, the culture supernates were extracted by a slightly modified method of Thomann and Hill,[13] and analysed for volatile fatty acids (VFA) and non-volatile fatty acids (NVFA). For the VFA, 0·2 ml of H_2SO_4 50%, 0·4 g of NaCl and 1 ml of methyl *tert*-butyl ether (MTBE) were added to 1 ml of acidified centrifuged supernate, or to a standard mixture of VFAs. Methylation of NVFAs was achieved by overnight incubation at room temperature of 1-ml volumes of acidified culture supernate with 0·2 ml of H_2SO_4 50% and 1 ml of methanol; 1 ml of water, 1 ml of MTBE and 0·4 g of NaCl were then added. In each case the tubes were mixed mechanically for 10 s and allowed to stand for 10 min before the top MTBE layer was removed for analysis. It was not found necessary to centrifuge the samples.

GC conditions. MTBE extracts (0·5 μl volumes) were analysed isothermally at 148°C on a column of SP1220 15%, H_3PO_4 1% on 100–120 mesh Chromosorb WAW in a Perkin-Elmer 8410 gas chromatograph fitted with flame ionisation detector, integrator and AS 8300 auto-sampler.

Identification schemes

Isolates were grown initially for 24–48 h in PPY medium or BM-CMB (trypticase 1%, proteose-peptone 1%, yeast extract 0·5%, NaCl 0·5%, sodium succinate 0·25%). The pH was adjusted to 7·4 and menadione (1 μg/ml), haemin (5 μg/ml) and L-cysteine HCl (0·075%) were added. Three volumes of this broth component were added to one volume of cooked meat particles,[14] and the complete medium was sterilised by autoclaving. Cultures were diluted in pre-reduced PPY medium to just visible turbidity before 0·9-ml inocula were added to predispensed substrates in tubes, or 30-μl volumes were inoculated onto agar media for the tests listed below, and incubated anaerobically for 2 days. If growth was slow or poor, incubation was extended up to a maximum of 5 days.

Tests for Fusobacterium spp. Lipase production (on egg yolk agar); motility; aesculin hydrolysis; indole production; fermentation of glucose, lactose, sucrose, maltose, mannose, trehalose, fructose and starch. In addition, selected isolates were tested for fermentation of galactose and utilisation of lactate and threonine.[15]

Tests for Bacteroides spp. Pigment production (on CBA plates); gelatin digestion; motility; nitrate reduction; urease production; aesculin hydrolysis, indole production; bile tolerance; fermentation of glucose, lactose, sucrose, maltose, xylose, rhamnose, trehalose, cellobiose, xylan, aesculin, raffinose and starch.

Tests were performed as described by Brown *et al.*[16] For fermentation tests a pH drop in the test substrate of greater than 0·5 units compared with the substrate-free control was considered a positive result. These tests have been successfully used in identification of clinical isolates, by comparison with results obtained with type cultures.

Statistical analysis

Spearman's rank correlation coefficients were calculated, where indicated in the text, to determine significance; the 5% level was considered significant.

Results

In periodontal disease in sheep, the first teeth lost tend to be the central incisors. For this reason, it was decided to sample from this area only, and from as many animals as possible by limiting samples to one per sheep as the animals would have to be dropped from the study on tooth loss. Seven visits were conducted over a period of 2·5 years, with no bacteriological samples being taken at visit number four. From the 15 sheep investigated, 1908 isolates of anaerobic bacteria from the subgingival plaque were analysed by GC for VFA and NVFA end-products of metabolism.

The incidence and range of the bacterial genera isolated is shown in table I, in descending order of prevalence. The majority of isolates were *Bacteroides* or *Fusobacterium* spp., comprising 49·2% and 17·5% respectively of the total number. Aerobic or facultative organisms comprised 10·3% of the total number of isolates, while 7·9% were unidentified either because they could not be recovered by subculture from the original isolate, or the GC profiles were not recognised. The incidence of most of the bacterial genera remained fairly constant, with only slight fluctuations, over the period of the longitudinal study. There were no significant differences in the types of organisms isolated from the flocks representing high, medium and low incidences of broken mouth periodontitis. There were significant negative correlations between the proportions of isolates that were *Lactobacillus*, aerobic organisms, and the combination of the less commonly isolated genera on the one hand and the visit number on the other. The latter group of less common genera comprised *Actinomyces, Propionibacterium, Leptotrichia* and *Bifidobacterium*; these were analysed cumulatively because their individual numbers were too low to test.

As the *Bacteroides* and *Fusobacterium* spp. accounted for nearly 70% of the organisms isolated, these were identified further by a series of biochemical and fermentation tests. Because some sheep were lost or removed from two of the farms for veterinary reasons, the remaining farm was selected for this part of the study: this one had an intermediate broken mouth problem and complete records.

The *Fusobacterium* isolates could be divided into three species—*F. nucleatum*-like (68·6%), *F. necrophorum* (29·6%) and *F. naviforme* (1·8%). However, the *F. nucleatum*-like isolates were unusual in that most of them fermented glucose and fructose more strongly than would be expected. This suggested that they may be more like the oral isolates obtained from monkeys, *F. simiae*,[17] or from man, *F. periodonticum*.[18] To try to verify this, 12 selected isolates were further tested for fermentation of galactose and utilisation of lactate and threonine (indicated by an increase in the production of propionate in the VFA GC profile when lactate or threonine is present in the growth medium). None of these isolates fermented galactose and was, therefore, not *F. periodonticum*. However, of the isolates tested, most utilised threonine and lactate to varying extents, indicating the possibility that they are more like the monkey isolate, *F. simiae*. As there are no clear cut definitions for interpretation of the tests for utilisation of lactate and threonine,[19] it was difficult to distinguish between *F. nucleatum*

Table I. Incidence and range of bacterial genera isolated from samples obtained from visits to three farms

Genus or group	Percentage of isolates representing each genus at visit no.						Total numbers of isolates	Percentage of all isolates
	1	2	3	5	6	7		
Bacteroides	37·7	45·7	54·7	59·3	44·8	52·7	1047	49·2
Fusobacterium	21·7	7·7	10·2	13·0	26·7	33·0	373	17·5
Lactobacillus	8·6	10·0	3·3	0·7	1·1	0·3	94	4·4
Clostridium	1·2	5·5	5·6	4·3	5·9	0·9	85	4·0
Anaerobic cocci	3·9	3·2	3·3	5·0	3·0	2·4	73	3·5
Eubacterium	0	6·4	1·6	2·7	2·2	0	49	2·3
Actinomyces	0	1·6	0·4	0	0	0	9	0·4
Propionibacterium	0·9	0·5	0	0	0	0	5	0·2
Leptotrichia	0	0·2	0·2	0	0·4	0	3	0·1
Bifidobacterium	0·3	0·2	0	0	0	0	2	0·1
Unidentified	11·3	4·5	10·0	2·3	10·7	8·8	168	7·9
Aerobes	14·5	14·5	10·7	12·7	5·2	1·8	219	10·3

and *F. simiae* by these criteria; for the purpose of this study we have defined these isolates as *F. nucleatum*-like.

The *Bacteroides* isolates were placed in groups according to their biochemical characteristics. Any group with fewer than five members, or in which the majority of the members were isolated from one subgingival plaque sample, were discounted. Eleven groups were obtained, which are outlined in table II.

It was difficult to assign the *Bacteroides* isolates to the currently recognised species. Groups 1, 3, 5 and 6 bear little resemblance to any described *Bacteroides* species, and possibly represent new (sheep-specific) species. Group 2 is similar to *B. oris* or *B. buccae*, whereas Group 7 resembles *B. disiens*. The asaccharolytic isolates, groups 8–11, could be described as *B. asaccharolyticus*-like, *B. nodosus*-like, *B. capillosus*-like and *B. gingivalis*-like, respectively.

The proportions of each *Bacteroides* group and *Fusobacterium* sp. isolated at each farm visit are shown in table III. The incidence of most of the *Bacteroides* groups fluctuated between visits. However, on examination of the overall trends, isolates in groups 3, 5, 6, 7, 8 and 11 appeared to increase in number as the study progressed. The numbers of *F. nucleatum*-like isolates were very variable but the numbers of *F. necrophorum* decreased with time. No statistical analysis of these results was made as the numbers of isolates involved at many of the sampling times were too low to give a statistically representative sample.

The degree of gingival inflammation, recorded at each farm visit, is represented by the gingivitis index (GI; fig. 1a); the pocket indices (PI) are shown in fig. 1b. During the period of the longitudinal bacteriological study (visits 1–7) the GI and PI rose steadily in two of the sheep (nos. 1 and 2) while the other three sheep showed more fluctuation and lower values.

The periodontal health of the sheep can be

Table II. Biochemical characteristics of the *Bacteroides* spp. isolated from sheep on the farm with an intermediate incidence of broken mouth periodontitis

Characteristic	Reaction of group no.										
	1	2	3	4	5	6	7	8	9	10	11
Pigment production	−	−	−	−	−	−	−	+	−	−	+
Gelatin hydrolysis	+	−	−(+)	+/−	+	−	+/−	+/−	+/−	−(+)	+
Nitrate reduction	−	−	−	−	−	−	−	−	−	−	−
Urease production	−	−	−	−	−	−	−	−	−	−	−
Aesculin hydrolysis	−	+	+(−)	−(+)	+	−	+/−	−	−	+	−
Indole production	−	−	+	−	−	−	−	+/−	−	−	+
Bile tolerance	−	−	−	−	−	−	−	−	−	−	−
Fermentation of:											
glucose	+	+	+	+	+	+	+	−	−	−	−
lactose	+	+	+	+	−	−	−	−	−	−	−
sucrose	−	+	+	+	+/−	+	−	−	−	−	−
maltose	−	+	+	+	+(−)	+	+(−)	−	−	−	−
xylose	−	+	−	−	−	−	−	−	−	−	−
rhamnose	−	+(−)	−	−	−	−	−	−	−	−	−
trehalose	−	−	−	−	−	−	−	−	−	−	−
cellobiose	−	+	−(+)	−	+	−	−	−	−	−	−
xylan	−	+/−	−	−	−	−	−	−	−	−	−
aesculin	−	+/−	−(+)	+/−	−(+)	−	−	−	−	−	−
raffinose	−	+	+	+(−)	+/−	−(+)	−	−	−	−	−
starch	−	+	+	+	+	+	+	−	−	−	−
GC profile	aPib ivLs	apLs	apls (iv)	apls (iv)	als (pibiv)	als (iv)	alS (iv)	apibb ivls	as (pib ivls)	as (pib ivl)	apbiv sph(1)
Number of isolates	60	47	35	13	27	13	23	39	27	9	22

+, >90% of strains positive; −, >90% of strains negative; +/−, 30–70% of strains gave each result; +(−) or −(+), 70–90% of strains gave the first result.

a, Acetic acid; P/p, propionic acid; ib, isobutyric acid; b, butyric acid; iv, isovaleric acid; ph, phenylacetic acid; L/l, lactic acid; and S/s, succinic acid. Upper case letters; ≥10 mmol/L; lower case letters, 0·2–9·9 mmol/L. Letters in parenthesis represent variable production.

Table III. Distribution of *Bacteroides* and *Fusobacterium* spp. isolated from sheep on a farm with an intermediate incidence of broken mouth periodontitis

Species or group	Percentage of all bacterial isolates at visit no.					
	1	2	3	5	6	7
Bacteroides group 1	2·7	11·4	17·3	3·3	4·0	2·5
2	2·7	0·7	4·0	20·0	2·7	1·7
3	0·7	5·7	4·0	6·0	0	8·3
4	2·0	1·4	1·3	1·3	0·7	2·5
5	0	1·4	1·3	0	11·3	5·0
6	0	0·7	0·7	1·3	2·0	5·0
7	0	0·7	2·0	2·7	4·7	6·7
8	0·7	4·3	3·3	3·3	8·7	7·5
9	0·7	2·1	4·0	7·3	2·7	1·7
10	0	0·7	1·3	2·0	1·3	0·8
11	0	0	4·7	0	3·3	8·3
F. nucleatum	15·3	0	0	4·0	29·3	35·0
F. necrophorum	10·7	7·9	6·0	6·0	0	3·3
F. naviforme	0·7	0	0·7	0	0·7	0
Total number of *Bacteroides* and *Fusobacterium* spp.	54	52	77	86	107	106

assessed by the dental index (DI: fig. 2), which represents the average of the indices for pocket depth, gingival inflammation, gum recession and tooth displacement measurements. Our previous experience has shown that a DI of greater than 10 predisposes to tooth loss. Two of the sheep (nos. 1 and 2) had rapidly increasing DI; sheep 2 had lost one central incisor at visit 7 (age 5 years) and sheep 1 had lost the central and second incisors by the end of the study (age 5 years). The other three sheep (nos. 3–5) appeared to have a more slowly progressing condition.

Unfortunately, we cannot compare the clinical status of the sampled sites in relation to their bacteriology because the samples were taken from sites distinct from the clinically monitored sites. This was to avoid any possible periodontal damage or bacteriological changes that may be caused by repeated probing for measurement of indices. However, pocket depths at the sampling sites were recorded and these followed the same trends as the overall pocket indices shown in fig. 1b.

When the *Bacteroides* and *Fusobacterium* isolates were compared, according to the periodontal status of the sheep from which they were isolated, only *Bacteroides* group 11, which consisted of the *B. gingivalis*-like isolates showed any differences (table IV). The majority of *B. gingivalis*-like organisms were isolated from the subgingival plaque of sheep with active broken mouth periodontitis (high dental indices); only one isolate of this type was from the sheep with a slowly progressing condition (low dental indices), and that was isolated during the last visit.

Discussion

The range of genera isolated in this study was similar to that obtained in a preliminary study of the anaerobic bacteria isolated from the subgingival plaque from sheep,[8] except that no *Veillonella* spp. were recovered as a *Veillonella* selective medium was not used. The incidence of *Eubacterium* spp. was higher than found previously, although this was mainly due to a high incidence of this genus in one single plaque sample. *Capnocytophaga* and *Eikenella* spp. have also been isolated in low numbers from subgingival plaque of sheep[6] but

Table IV. Distribution of *B. gingivalis*-like isolates (Group 11) from sheep with high and low final cumulative dental indices

Dental index*	Percentage of total bacteria isolated at visit no.					
	1	2	3	5	6	7
High	0	0	11·7	0	8·3	15·0
Low	0	0	0	0	0	1·7

* Dental index was considered high when the final cumulative dental index was ≥ 10 and low when < 10 (see fig. 2). Data are for the animals included in figs. 1 and 2.

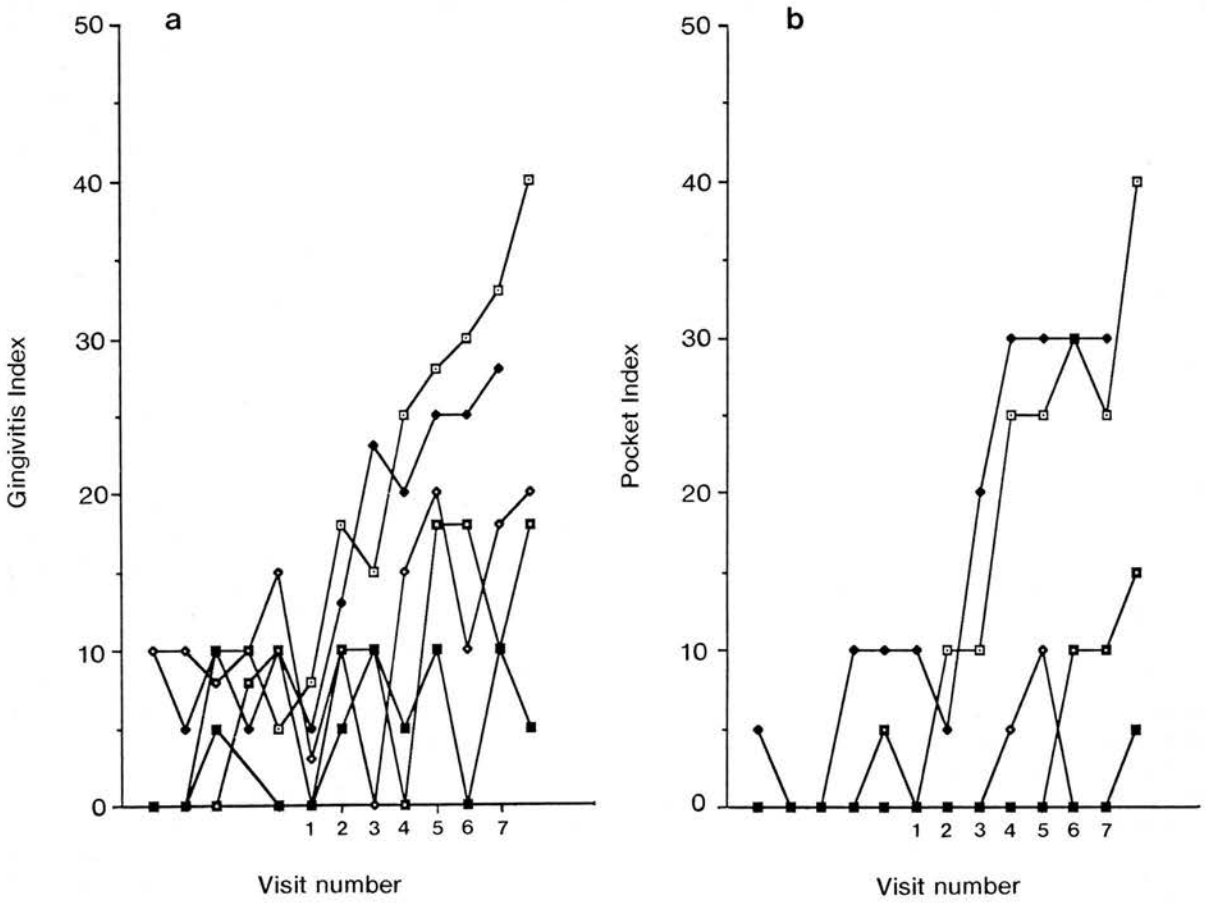

Fig. 1. Changes in (**a**) gingivitis indices and (**b**) pocket indices in five sheep with sequential visits to a farm with an intermediate incidence of broken mouth periodontitis, over a period of 4·5 years. Bacteriological samples were taken at visits 1–7, over a period of 2·5 years. Sheep 1 -⊡-; sheep 2 -♦-; sheep 3 -■-; sheep 4 -◇-; sheep 5 -■-.

these organisms were not found in this study. The incidence of each of the more commonly isolated genera or groups (*Bacteroides, Fusobacterium, Clostridium*, anaerobic cocci and *Eubacterium*) remained relatively stable over the period of the longitudinal study. Only *Lactobacillus* spp. and the less commonly isolated genera were seen to reduce in numbers over the 2·5 year project.

The three farms used were chosen initially for the different records of periodontal disease so that a range of periodontal conditions could be covered throughout the longitudinal study. However, the sheep used on each individual farm gave a range of periodontal disease involvement. In the farm chosen for detailed study of the *Fusobacterium* and *Bacteroides* isolates, two of the sheep had rapidly progressive periodontitis culminating in tooth loss, while the dentition of the other three remained relatively healthy over the 2·5 year study.

The dental health indices (GI, PI and DI) and incidences of bacterial genera and bacterial groups fluctuated considerably during the study. This confirmed the results obtained in a year-long study of the clinical development of broken mouth in a single flock of sheep[10] and could reflect the episodic nature thought to occur with periodontitis in man[20,21] and in sheep.[22] Broken mouth periodontitis in sheep has also been shown to have seasonal variation[23] and may be affected by nutritional and environmental factors.[10] The sheep used in this study were hill sheep, and only readily available for sampling three times a year. There was no control over environmental factors, and diets may have varied, due to increased feeding of pregnant ewes, especially those expected to produce twins.

Fusobacterium spp. are found commonly in human subgingival plaque, and *F. nucleatum* is the most frequently detected species.[24] This species is

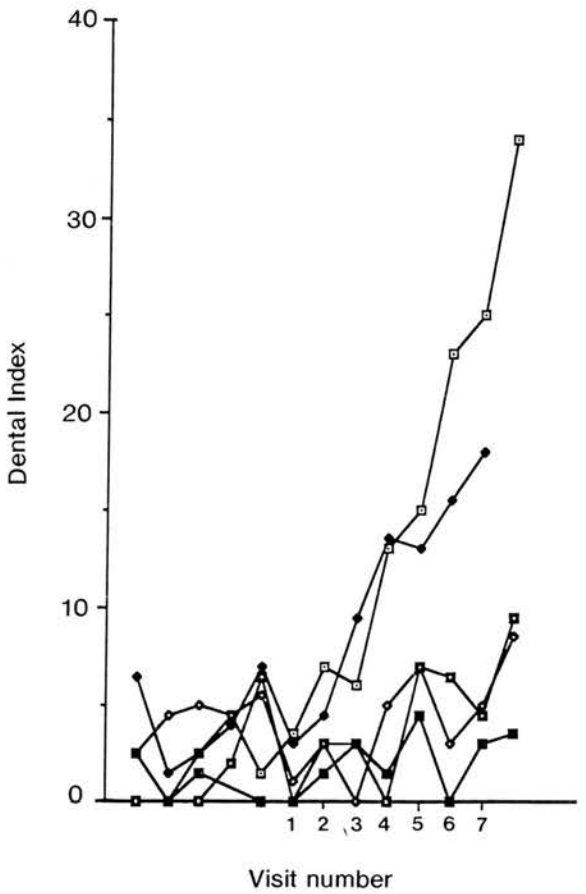

Fig. 2. Changes in dental indices in five sheep with sequential visits to a farm with an intermediate incidence of broken mouth periodontitis, over a period of 4·5 years. Bacteriological samples were taken at visits 1–7, over a period of 2·5 years. Sheep 1 –□–; sheep 2 –◆–; sheep 3 –■–; sheep 4 –◇–; sheep 5 –■–.

associated with both active and inactive periodontal sites in man[25,26] and it is thought that this might indicate a heterogeneity within the species.[24] Similarly, in this study there was no difference in the incidence of *F. nucleatum*-like isolates between the two categories of periodontal status described.

The *Fusobacterium* isolates from sheep comprised three species biochemically similar to the human types. However, further examination of selected organisms from the *F. nucleatum*-like group indicated that some of these may be *F. simiae*. These two species have been shown to be closely related by DNA homology studies.[27] Clearly the isolates from sheep merit a more detailed investigation.

In a bacteriological study with sheep allocated to three groups according to degree of periodontal destruction, *F. necrophorum* and *F. nucleatum* were found commonly.[6] In addition, *F. nucleatum* Type II (separated from Type I by colony morphology on selective medium)[28] correlated with disease severity. Indeed, a statistically significant increase in Type II isolates with increasing levels of gingivitis in human periodontal disease has been demonstrated[29] although the two types appeared to be identical by double diffusion in agar and similarity of polyacrylamide gel electrophoresis protein patterns. In the study presented here, it was difficult to distinguish between colony types (results not shown) and, because of the possibility that some of the isolates might have been *F. simiae*, this was not investigated further.

A study of *Fusobacterium* spp. isolated from bite wounds in cats indicated that *F. russii* predominates in the oral *Fusobacterium* population with smaller amounts of *F. necrophorum, F. naviforme* (found in low numbers in this study) and *F. nucleatum* being isolated.[19] Those isolated were shown to be genetically distinct from human oral strains.[30]

In studies of human periodontitis, spirochaetes have been found more frequently and in higher numbers in diseased sites compared with healthy sites.[31] Spirochaetes were observed in all plaque samples in this study by dark ground microscopy, and also in other studies.[10,32] Our attempts to grow sheep spirochaetes (not reported here) were largely unsuccessful. However, spirochaetes from monkey periodontal pockets have been shown to have different nutritional requirements from human isolates[33] and this may account for the lack of success in this study.

Many of the *Bacteroides* spp. isolated in this study were very fastidious, and grew poorly in pure culture on solid media. In many cases growth was enhanced by deliberately seeding the agar media, close to the *Bacteroides* inoculum, with an aerobic organism. Growth was enhanced near to the stimulating organisms, particularly with the black-pigmented *Bacteroides* where pigment production was just detected at the opposing edge. This implies that these isolates require additional nutrients not present in the media, and may be indicative of the dissimilarity of these isolates from human strains.

The sheep *Bacteroides* isolates could be placed in 11 broad categories by the tests used in this study. Groups 1, 3, 5, and 6 are likely to be new species of *Bacteroides*. However, a full characterisation of these isolates with guanine plus cytosine content of the DNA and DNA–DNA homology studies would have to be performed to confirm this. Under certain growth conditions a small proportion of the cells of two isolates of Group 4 were motile and this merits further investigation. Group 7 was like *B. disiens*.

Although this organism is commonly isolated from the vaginal flora, it has also been recognised as a common anaerobic isolate from bite-wound infections.[34]

Although the isolation frequency of several groups of *Bacteroides* spp. and *F. nucleatum*-like organisms increased during the study, only the *B. gingivalis*-like isolates appeared to be associated with active periodontal disease. No statistical analysis was carried out on these data as the large number of zero values made this difficult, and the number of sheep in the groups (two in the high index group and three in the low index group) was considered too low to provide significant results.

Frisken *et al.*[5,6] have also shown increased incidence of total black-pigmented *Bacteroides* species and, in particular, *B. gingivalis* in periodontally affected sheep. However, the incidence of *B. gingivalis*-like organisms was much lower than that found in this study (up to 15% of the total number of anaerobes in periodontally affected sheep) and differences were not statistically significant. This difference could be due to the longitudinal nature of this study, with increased chances of sampling during active episodes of disease progression, or to the use of different isolation media.

B. gingivalis is commonly thought to be an important periodontopathic component in periodontitis of man, and a correlation between degree of inflammation and quantity of *B. gingivalis* in subgingival plaque has been shown.[35,36] Thought to be the most virulent of the black-pigmented *Bacteroides*,[37] *B. gingivalis* has extensive proteolytic properties[38] and its lipopolysaccharide, although low in toxicity, has been shown to mediate bone resorption *in vitro*[39] and *in vivo*.[40] Isolates from sheep have been shown to have enzyme activities similar to human isolates, including a trypsin-like activity.[41] Oral *B. gingivalis*-like isolates have been described in other animals—cats, dogs, raccoons, jaguars and squirrel monkeys.[42–44] From our study, *B. gingivalis*-like isolates appear also to play a role in broken mouth in sheep.

The results of this study indicate some similarities and also some differences in the periodontal flora of man and sheep. This merits further investigation, with more animals, and further characterisation of the isolates obtained.

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Characterisation of *Bacteroides* from sheep periodontal disease by SDS–PAGE of outer membrane proteins

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1. SUMMARY

Isolates of *Bacteroides* species obtained from a longitudinal study of developing periodontal disease in sheep were analysed by SDS–PAGE. Protein profiles of Sarkosyl-insoluble outer membrane extracts were compared within groups of isolates which had already been defined by conventional biochemical techniques. Heterogeneity was exhibited within most groups. Isolates of *B. gingivalis* and *B. asaccharolyticus* shown to be similar to human isolates by conventional biochemical tests, gave different protein profiles from the respective type cultures. The sheep *B. gingivalis*-like isolates were however homogeneous, while the *B. asaccharolyticus*-like organisms could be divided into 3 subgroups. SDS–PAGE appears to be a useful tool for the examination of bacterial flora and recognition of subgroups or subspecies.

2. INTRODUCTION

Periodontal disease in sheep (broken mouth) is a chronic inflammatory condition of the supporting tissues of the teeth and shows many similarities to periodontal disease in man [1,2]. However, little is known about the bacteriology of the sheep condition, although the genera of bacteria isolated from sheep subgingival plaque have been shown to be similar to those obtained from man [3,4]. In a longitudinal study of the subgingival anaerobic bacteria isolated from sheep during the development of broken mouth periodontitis, almost half of the isolates belonged to the genus *Bacteroides*. Initial characterisation divided these isolates into 11 groups, of which at least 4 are likely to be new sheep-specific species [5].

Classical identification of microbial species has been based on morphological, biochemical, serological, toxigenic and genetic characteristics. More recently, cellular protein profiles, obtained by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) have been employed to distinguish closely related species, or strains within a species. Genera studied have included *Arthrobacter* [6], cariogenic *Streptococcus* species [7], *Mycoplasma* [8], *Clostridium* [9], *Bifidobacterium* [10] and *Veillonella* [11]. PAGE protein profiles also distinguish between *Bacteroides* species, subspecies and reveal minor strain differences [12,13]. Use of this method has been suggested for studying bacterial flora [14] and for rapid identification of Gram-negative species involved in periodontal disease [15] and in particular, asaccharolytic non-

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pigmented species which are difficult to differentiate from each other [16]. This method is particularly useful for distinguishing *B. oris* and *B. buccae* [17].

Most of the studies utilise PAGE of total cell protein. In this study detergent-insoluble outer membrane proteins were employed to reduce complexity of the profiles, and to include proteins likely to be antigenic in vivo. Protein profiles of the major sheep *Bacteroides* groups, previously described [5], were compared for similarity or heterogeneity amongst the isolates, and where possible to compare the sheep isolates with the relevant type culture.

3. MATERIALS AND METHODS

3.1. Organisms

Representative isolates of 8 groups of *Bacteroides* species isolated during a longitudinal study of developing periodontal disease in sheep, described elsewhere [5] were utilised. They were divided into groups according to their reactions in the following tests: gas chromatographic analysis of volatile and non-volatile fatty acid metabolic end-products; pigment production; gelatin digestion; nitrate reduction; urease production; aesculin hydrolysis; indole production; bile tolerance; fermentation of glucose, lactose, maltose, xylose, rhamnose, trehalose, cellobiose, xylan, aesculin, starch.

Type cultures (all isolated from humans) included for comparisons were, *B. disiens* VPI 8047, *B. asaccharolyticus* NCTC 9337 and *B. gingivalis* ATCC 33277. These were obtained from NCTC, London.

3.2. Growth conditions

Organisms were grown in PPY medium (Proteose-peptone, yeast extract medium with additional growth factors [18]). Batches of medium (500 ml) were inoculated with overnight starter cultures (50 ml) and incubated in an anaerobic cabinet (Forma Scientific, Ohio) in an atmosphere of 80% N_2, 10% H_2 and 10% CO_2 at 37°C for 48 h.

3.3. Outer membrane preparation

Cells were harvested and washed once in phosphate buffered saline (pH 7.4; PBS). The bacteria were resuspended in 5 ml distilled water and lysed by sonication, on ice (MSE Soniprep 150) at an amplitude of 8–10 μm until more than 90% of the cells were broken (up to five 1 min bursts with 30 s intervals between bursts). Unbroken cells were removed by centrifugation at $5000 \times g$ for 10-min. The lysed cell supernatant was mixed with 7% sodium *N*-lauroyl sarcosinate (Sarkosyl; Sigma) to a final concentration of 0.7%. Outer membrane preparations were sedimented by centrifugation at $50\,000 \times g$ for 1 h, washed with distilled water and finally resuspended in 0.5 ml distilled water by repeated passage through a 26-gauge needle. Samples were stored at −20°C.

3.4. SDS–PAGE

The protein content of the preparations was determined by the Lowry method [19]. Samples were diluted to a protein concentration of 400 μg/ml, mixed with an equal volume of sample buffer and boiled for 3 min. Gels were prepared, run and stained as described by Poxton [20].

4. RESULTS AND DISCUSSION

The isolates in this study could not easily be assigned to currently recognised species of *Bacteroides*, and were grouped according to their biochemical characteristics. Groups 1, 3, 4 and 5 are thought to be new (sheep-specific) species, while group 2 is similar to *B. oris* or *B. buccae* and group 7 resembles *B. disiens*. The asaccharolytic isolates, groups 8 and 11, could be described as *B. asaccharolyticus*-like and *B. gingivalis*-like, respectively [5]. Isolates were chosen to represent the 3 different farms from the longitudinal study and to cover the time span of the study.

Sarkosyl-insoluble extracts were run on SDS–PAGE gels, and the protein profiles compared. Groups 7, 8 and 11 were compared with the reference strains, *B. disiens* VPI 8047, *B. asaccharolyticus* NCTC 9337 and *B. gingivalis* ATCC 33277, respectively.

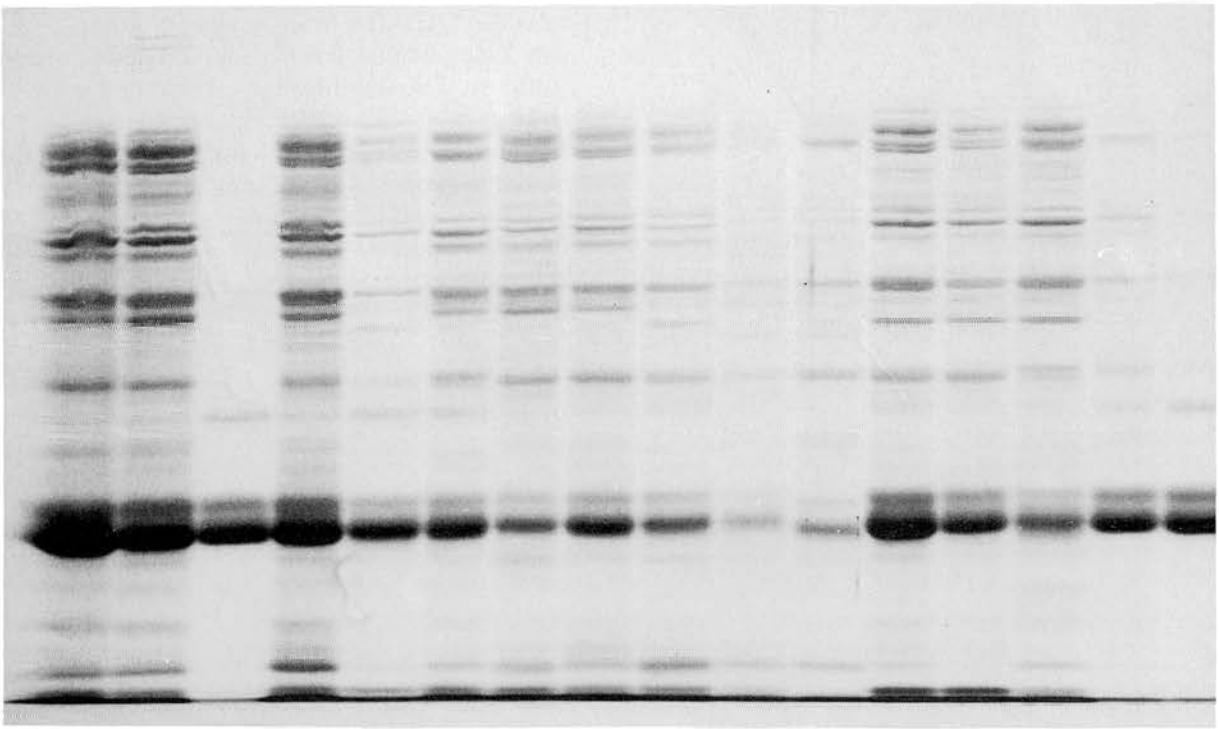

Fig. 1. SDS–PAGE profiles of Sarkosyl-insoluble fractions obtained from *Bacteroides* group 1 isolates.

The table lists the number of distinct protein profiles obtained for each *Bacteroides* group. Only 2 of the 8 groups are clearly homogeneous and likely to represent a single species. The other groups all have more than one protein profile and with group 2, each isolate gave a different protein profile. These results are further illustrated by the figures. Fig. 1 represents group 1 *Bacteroides*, which all gave similar protein profiles. The profiles of group 3, which can be subdivided into 2 groups are represented in Fig. 2. The sheep *B. asaccharolyticus*-like isolates could be divided into 3 groups according to protein profiles (Fig. 3) and were distinct from the reference strain.

The results obtained for the *B. gingivalis*-like isolates (group 11) are not shown, as the Sarkosyl-insoluble extracts were not stable and did not store well, possibly due to proteolytic activity. However these isolates gave 1 distinct profile but again differed from the type culture. These results indicate that the asaccharolytic sheep species are distinct from the human ones. It has already been shown that human *B. gingivalis* isolates are serologically distinct from isolates obtained from other animals (cats, dogs, jaguars and

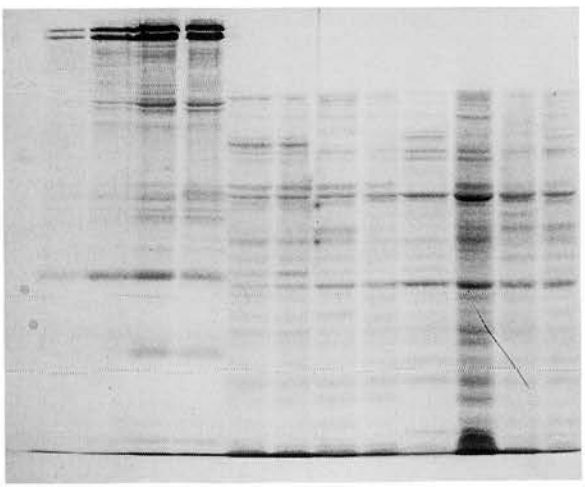

Fig. 2. SDS–PAGE profiles of Sarkosyl-insoluble fractions obtained from *Bacteroides* group 3 isolates.

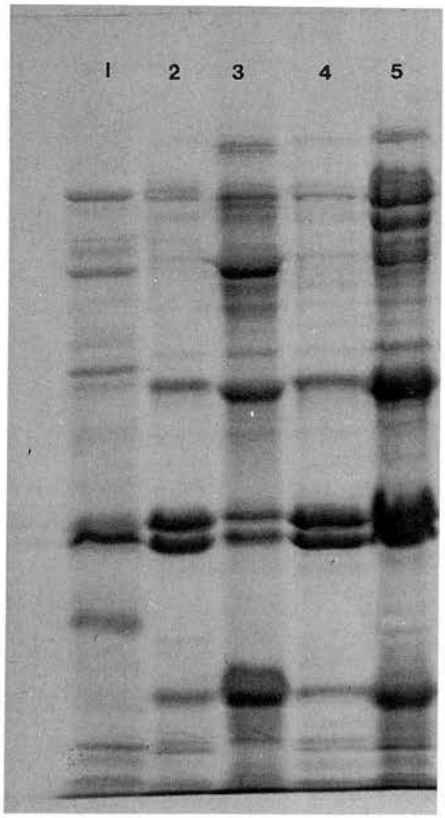

Fig. 3. SDS–PAGE profiles of Sarkosyl-insoluble fractions obtained from *Bacteroides* group 8 isolates (*B. asaccharolyticus*-like) compared with a reference strain (*B. asaccharolyticus* NCTC 9337; Track 1).

Table 1

The distribution of protein profiles obtained by SDS–PAGE of Sarkosyl-insoluble extracts of eight different groups of sheep oral *Bacteroides* isolates

Bacteroides Group [a]	Number of isolates	Number of distinct protein profiles
1	37	1
2	6	6
3	17	2
4	19	5
5	5	3
7	5	3
8	29	3
11	11	1

[a] The *Bacteroides* groups have been described in detail previously [5].

raccoons [21]). The heterogeneity of groups 2, 4, 5 and 7 suggest that biochemical tests alone are not sufficient for identification of sheep *Bacteroides* isolates. Use of an electrophoretic technique in conjunction with classical taxonomic approaches has been proposed to differentiate closely related oral species [22]. This study indicates that SDS–PAGE of Sarkosyl-insoluble preparations would be useful for the screening of sheep oral bacterial flora.

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The association on SDS–polyacrylamide gels of lipopolysaccharide and outer membrane proteins of *Pseudomonas aeruginosa* as revealed by monoclonal antibodies and Western blotting

(*Pseudomonas aeruginosa*; monoclonal antibodies; Western blotting; lipopolysaccharides; outer membrane proteins)

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1. SUMMARY

Monoclonal antibodies raised against single serotype components of a *Pseudomonas aeruginosa* vaccine have been shown to bind to the O antigen region of lipopolysaccharide (LPS). Outer membrane (OM) proteins, prepared by detergent treatment of envelope fractions and by EDTA/sonication treatment of whole cells, were separated on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), electrophoretically transferred to nitrocellulose membrane and reacted with LPS-specific monoclonal antibodies. The patterns produced revealed that many of the protein bands were in fact protein–LPS complexes.

2. INTRODUCTION

The OM of Gram-negative bacteria consists of LPS, phospholipids and a range of OM proteins. Several methods have been developed for the preparation of OM. One method depends on the physical separation of inner and outer membrane on a density gradient [1], while possibly the most commonly used method involves the treatment of envelope fractions with detergents which selectively leave the OM as an insoluble pellet. The method developed by Schnaitman [2] makes use of the detergent Triton X100 and has been used widely for *Escherichia coli* [3] and for other Gram-negative bacteria [4]. A similar method, developed by Filip et al., using the detergent Sarkosyl, has also been employed [5]. Methods based on extraction of whole cells with EDTA have also been used, e.g., [6].

The OM of *P. aeruginosa* has been investigated by several workers. For example, Hancock and coworkers [7] used a gradient centrifugation method for its preparation, while Meadow et al. [8] used Triton. Both methods gave highly similar patterns of OM proteins on SDS–polyacrylamide gels.

The LPS of *P. aeruginosa* is the type-specific antigen of the species, and there are at least 16 known serotypes [9]. In a separate study, monoclonal antibodies have been prepared to 2 serotypes of *P. aeruginosa* [10]. Several of these monoclonal antibodies have been shown to be serotype-specific and to react with the O antigen region of

the LPS (G.R. Barclay et al., unpublished). In the present study, we used LPS-specific monoclonal antibodies as probes to demonstrate the distribution of LPS molecules among the OM proteins on SDS-polyacrylamide gels.

3. MATERIALS AND METHODS

3.1. Bacterial strains and growth conditions

P. aeruginosa Habs serotypes 1 and 4 were kindly supplied by Dr. R.J. Jones, MRC Vaccine Research Laboratories, Medical School, Birmingham, U.K. Overnight cultures were grown in 1-l volumes of Oxoid No. 2 Nutrient Broth, in 2-l conical flasks in an orbital incubator at 37°C for 16 h. Log-phase cultures were grown from a 10% overnight culture inoculum under the same conditions as above, but for 4 h.

P. aeruginosa serotype 1 was also grown in [^{32}P]orthophosphate in an attempt to label the LPS. A synthetic medium based on that of Robert-Gero et al. [11] was used. An overnight culture in Nutrient Broth (1 ml) was used to inoculate 200 ml of synthetic medium, and was incubated for 16 h. This culture was added to 800 ml of fresh medium and incubated for a further 1 h. Cells were harvested, washed once in saline and resuspended in 100 ml synthetic medium, in which the phosphate had been replaced with 50 mM Hepes buffer, pH 7.2, containing 100 μCi of ^{32}P-labelled orthophosphate (Amersham). After a further 2.5 h, incubation cells were harvested and outer membranes separated by the Sarkosyl method (see below).

3.2. Preparation of LPS

LPS was extracted from washed, freeze-dried bacteria harvested from an overnight culture by the 45% (w/v) aqueous phenol method developed by Westphal and Luderitz [12]. The LPS was washed and purified by 2 cycles of centrifugation at 100 000 $\times g$ for 3 h, and stored freeze-dried.

3.3. Preparation of OM proteins

Log-phase bacteria (4 l) were harvested by centrifugation at 16 000 $\times g$ for 10 min and washed once in 0.01 M Hepes buffer, pH 7.4, then suspended in 40 ml Hepes. Cells were broken by passage through a French pressure cell (Aminco, Silver Springs, MD, USA) at 6000–7000 p.s.i. (42–48 MPa). Unbroken cells were removed by 2 cycles of centrifugation at 6000 $\times g$ for 10 min. The suspension was divided into two; one part to be used for Triton extraction and the other for Sarkosyl.

Triton treatment was by the method of Diedrich et al. [13]. $MgCl_2 \cdot 6H_2O$ was added to a final concentration of 1 mM. Envelopes were sedimented at 50 000 $\times g$ for 1 h. The pellet was resuspended in 20 ml Hepes buffer, containing 2% (v/v) Triton $\times$100, and incubated in a water-bath for 10 min at 23°C. The OM proteins were sedimented at 50 000 $\times g$ for 1 h, washed once in water and resuspended in 2 ml water.

Sarkosyl treatment was by a modification of the method of Filip et al. [5]. Sarkosyl (30% w/v solution of sodium *N*-lauroyl sarcosinate (Sigma Chemical Co.)) was added to the broken cell suspension to a final concentration of 0.7% (w/v) sodium *N*-lauroyl sarcosinate. The suspension was centrifuged at 50 000 $\times g$ for 1 h, and the pellet of OM was washed once in water and finally suspended in 2 ml water (0.5 ml in the case of the ^{32}P-labelled extract).

EDTA treatment was by the method of Poxton and Brown [6].

3.4. Preparation of monoclonal antibodies

The preparation and characterisation of monoclonal antibodies to *P. aeruginosa* vaccine (PV) is the subject of a complete paper which is in preparation (Barclay et al., unpublished). Briefly, mice were immunised with the serotype 1 or 4 component of polyvalent *Pseudomonas* vaccine (PPV) [14]. Hybridomas for each serotype were prepared by standard techniques [15]. The majority of the hybridomas obtained produced monoclonal antibodies which were serotype-specific and were shown to bind to the O antigen region of the LPS. Representatives of these monoclonal antibodies were used in the present study.

3.5. SDS-polyacrylamide gel electrophoresis

This was performed on 10% slab gels with the buffer system of Laemmli [16], by the method of

Poxton and Brown [6]. Protein (50 μg), LPS (12.5 μg carbohydrate) or ^{32}P-labelled Sarkosyl extract (10^6 cpm) were run. Gels were stained with Coomassie blue [17], silver [18] or used for electroblotting. Urea (4 M) was added to some gels.

3.6. Electroblot transfer and enzyme immunoassay

This was essentially by the method of Towbin et al. [19] with BioRad Immunoblot immunoassay, as described previously [20].

3.7. Carbohydrate and protein assays

These were by the methods of Dubois et al. [21] and Lowry et al. [22], respectively.

3.8. Autoradiography

Electroblots of ^{32}P-labelled Sarkosyl extract run on SDS–PAGE and X-ray film (Fuji) were placed in a cassette fitted with intensifying screens and exposed for 64 h at −70°C.

4. RESULTS AND DISCUSSION

SDS–polyacrylamide gels of the OM protein preparations, whether prepared by Triton or Sarkosyl, were almost identical when stained with Coomassie blue (Fig. 1a and b), and those prepared from *P. aeruginosa* serotype 1 were extremely similar to those from serotype 4 (Fig. 1d). The major OM proteins are labelled to conform with the scheme of Mizuno and Kageyama [23]. OM prepared by EDTA/sonication are significantly different, but most of the bands are present, although in different proportions (Fig. 1c). Silver-stained gels of the LPS preparations showed the characteristic ladder pattern, each band differing in M_r by one repeating unit of O antigen. An example of a silver-stained gel of *P. aeruginosa* serotype 1 is shown in Fig. 1e.

Unstained gels similar to those described above were transferred to nitrocellulose membranes and probed with a series of monoclonal antibodies. 2 Monoclonal antibodies (Nos. 1.5 and 1.7) raised against the serotype 1 vaccine, and previously shown (by ELISA and inhibition of ELISA) to be specific for the O antigen region of the LPS (Barclay et al., unpublished), were reacted with

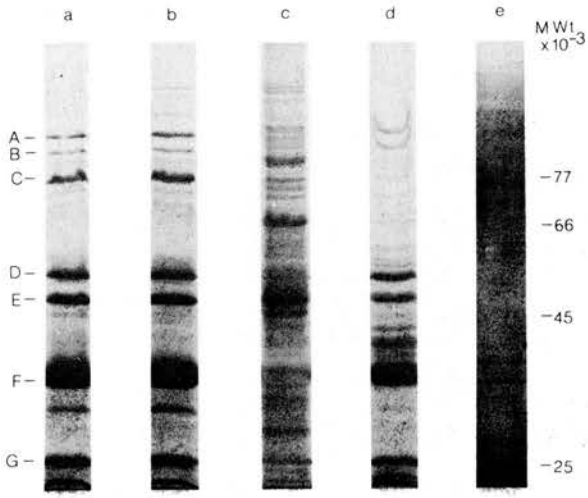

Fig. 1. SDS–polyacrylamide (10%) gels of (a) Sarkosyl, (b) Triton, and (c) EDTA preparations from *P. aeruginosa* serotype 1; (d) Sarkosyl preparation of serotype 4, stained with Coomassie blue; (e) LPS of serotype 1, stained with silver.

LPS, Triton, Sarkosyl and EDTA preparations. With the serotype 1 LPS preparation, both monoclonal antibodies produced the characteristic ladder pattern. This was similar to the silver-stained gel, except that there was no stain at the bottom of the gel at the position corresponding to rough LPS. With the Triton and Sarkosyl preparations, a different pattern was produced. This closely followed the pattern of the Coomassie blue-stained gel. Most protein bands, with the notable exception of the porin (F) band, were stained. There was no significant difference between the Triton and Sarkosyl preparations. The EDTA preparation gave a pattern similar to the LPS pattern at the lower part of the gel, but was more like the protein pattern for the higher M_r bands. Fig. 2 shows examples of the patterns produced when monoclonal antibody 1.5 was reacted with (a) Sarkosyl, (b) EDTA and (c) LPS preparations of *P. aeruginosa* serotype 1; and (d) Sarkosyl and (e) LPS preparations of serotype 4. These reactions were specific for serotype 1. Monoclonal antibodies shown to be serotype 4-specific showed the opposite pattern, reacting only with the serotype 4 extracts. Fig. 2f shows the pattern produced when a Sarkosyl extract of serotype 4 was reacted with a serotype 4-specific monoclonal antibody. The pat-

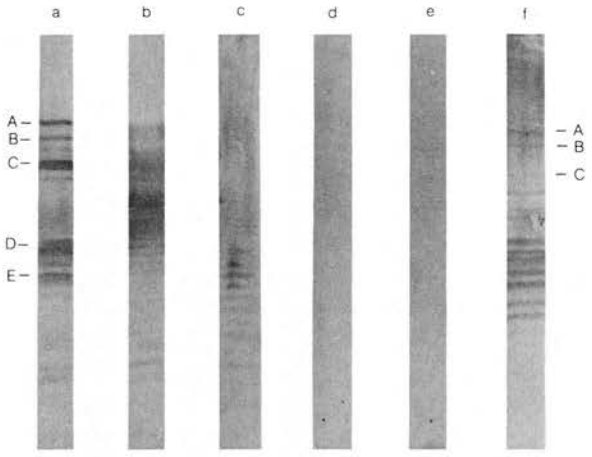

Fig. 2. Western blots of (a) serotype 1 Sarkosyl preparation; (b) serotype 1 EDTA extract; (c) serotype 1 LPS; (d) and (f) Serotype 4 Sarkosyl extracts; and (e) Serotype 4 LPS. Tracks (a)–(e) were reacted with the monoclonal antibody specific for the LPS of *P. aeruginosa* serotype 1, and track (f) was reacted with the monoclonal antibody specific for serotype 4.

tern produced when this monoclonal antibody was reacted with homologous LPS gave a pattern identical to that in Fig. 2c. If 4M urea was incorporated into the stacking and separating gels, no differences in the patterns described above were observed.

Several of the monoclonal antibodies produced in each fusion were not species-specific, but cross-reacted in the screening enzyme-linked immunosorbent assay with vaccine preparations from other serotypes of *P. aeruginosa*. These antibodies were not directed against LPS, and 2, one from each fusion, were used as controls in the above series. No reactions were observed between these cross-reactive monoclonal antibodies and either LPS preparation. The antibodies only produced reactions when the OM extracts, but not LPS, were spotted directly onto nitrocellulose membranes and treated with the immunoblot reagents. After separation on SDS–PAGE no lines were seen. This suggested that these cross-reactive monoclonal antibodies were directed against proteins that were denatured irreversibly by the electrophoresis and/or transfer procedures.

Monoclonal antibodies directed against the LPS of *P. aeruginosa* have allowed us to define specifically the location of LPS molecules among the OM proteins after separation on SDS–polyacrylamide gels. It is perhaps not surprising to find that the LPS is associated with some of the OM proteins in OM prepared by detergent insolubility, and is not simply revealed as the ladder pattern superimposed on the OM protein pattern. In the intact OM, LPS and proteins are in intimate association. The solubilisation procedure used on the samples prior to electrophoresis involved heating to 100°C for 6 min in 2% SDS and 1% 2-mercaptoethanol, and in some experiments urea was incorporated in the gels; the association withstands these treatments. These findings are in apparent contradiction to those for *Salmonella* spp., where OM proteins have been shown not to be associated with LPS [24]. In that study, however, OM were prepared by EDTA/sonication. In our similar preparation from *P. aeruginosa,* the subsequent blot with LPS-specific monoclonal antibodies showed a pattern more similar to that of pure LPS, but still with some high-M_r LPS-associated protein.

In a control experiment, we attempted to label the LPS with radioactivity by incubating growing cells with [^{32}P]orthophosphate (see MATERIALS AND METHODS). Autoradiographs of the Sarkosyl extracts showed a picture similar to that obtained with LPS-specific monoclonal antibodies in a Western blot. Most of the ^{32}P had been incorporated into phospholipid, which ran near to the front of the gel, but weaker bands corresponding to protein bands could be seen. No ladder pattern was apparent.

We suggest that those methods for preparing OM based on the insolubility in detergents work by selectively leaving, and perhaps encouraging, protein/LPS complexes which are highly resistant to disaggregation.

Our findings have important implications. When undertaking studies on antibodies directed against OM antigens of *P. aeruginosa,* and possibly of other Gram-negative bacteria, Western blotting should be used with caution. The OM proteins need not necessarily be pure proteins, but protein/LPS complexes and antibodies detected may be against either antigen. It has only been possible to demonstrate this association conclusively with monoclonal antibodies.

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Changes in Anti-Endotoxin-IgG Antibody and Endotoxaemia in Three Cases of Gram-Negative Septic Shock

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Circulating endotoxin levels and IgG antibodies to a range of Gram-negative bacterial lipopolysaccharides (LPS) (endotoxins) of different sizes and structures were measured daily in three cases of septic shock. There was an inverse relationship between endotoxin levels and cross-reactive antibodies to the core glycolipid (CGL) region of lipopolysaccharide. This suggests that antibody to LPS-CGL was initially consumed by a superabundance of endotoxin, and that a resurgence of intrinsic anti-LPS-CGL antibody levels may be associated with a reduction of circulating endotoxin. The implications of these findings for passive antibody therapy of septic shock are discussed.

Key words: LPS, anti-LPS, core glycolipid, ELISA

INTRODUCTION

The relationships between bacteraemia, endotoxaemia, and septic shock remain unclear. Septic shock commonly occurs in patients with Gram-negative bacteraemia but may also develop in patients with Gram-positive or fungal infections or even when no obvious source of infection can be demonstrated [1–5]. Positive blood cultures have been reported to be detectable in as few as one-third of patients in septic shock [4]. Endotoxin appears to play a central role in the pathogenesis of septic shock [6,7]. In cases of Gram-negative bacteraemia a source of endotoxin is apparent: in other cases it presumably originates from the endogenous Gram-negative bacteria in the gut [8,9].

Recent reviews of a variety of studies of the role of passive antibody therapy in treatment of septicaemia and septic shock have concluded that while antibodies to the

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lipopolysaccharide (LPS) endotoxins of Gram-negative bacteria have potential for immunotherapy in septic shock, the mechanism by which anti-LPS antibodies act and their specificities for regions of the LPS molecule associated with therapeutic activity remain unclear [10–12]. Thus, parameters for selecting antibodies with high therapeutic activity have not been defined.

Serotype-specific antibodies to the O-polysaccharide outer chains of LPS of different bacteria have been shown to be bactericidal in animal protection models [13–15], but may not be protective in clinical bacteraemia [16]. Further, because of inherent difficulties or delays in detecting bacteria and determining serotypes in patients, serotype-specific immunotherapy may be inappropriate in septic shock. Successful immunotherapy is likely to require cross-reactive antibodies to structures common to LPS from different bacteria, without reference to serotype. Conserved structural regions with which cross-reactive anti-LPS antibodies interact are available within the CGL region of LPS [17–20]. The CGL terminates in lipid A, which, on its own, expresses most of the toxic properties of endotoxin [18]. Anti-LPS-CGL cross-reactive antibodies might therefore neutralise endotoxic activities of free LPS by binding to and masking biologically active sites, as well as promoting opsonisation and clearance of LPS. Such antibodies might not be efficient as bactericidal antibodies if LPS-CGL structures are poorly exposed on bacterial surfaces and may be less important in bacteraemia than serotype-specific antibodies to the more exposed polysaccharide chain of LPS. This remains to be clearly established.

It is now known that there are differences in CGL structures between LPS from different Gram-negative bacteria [17–20], and it is not apparent whether truly pan-reactive anti-LPS-CGL antibodies exist or could be achieved by immunisation or whether any such antibodies would be therapeutically active. High endogenous levels of anti-LPS antibodies [21], and, in particular, anti-LPS-CGL antibodies [16,22], appear to be associated with protection against bacteraemia and septic shock in at-risk patient groups. However, information on the dynamics of anti-LPS antibody levels, their range of specificities, and their possible interaction with circulating endotoxin during septic shock is not available.

We have developed a sensitive immunoassay for anti-LPS IgG antibodies employing a range of complete and incomplete (CGL-component) LPSs, which can demonstrate anti-LPS-CGL cross-reactivity patterns in sera [20]. We have used this assay to determine sequential changes in anti-LPS antibodies and endotoxin levels in sera from three cases of septic shock and have found evidence of apparent interaction between certain anti-LPS antibodies and serum endotoxin.

CASE REPORTS

All patients admitted to the Intensive Therapy Unit (ITU) were placed on a continuous prophylactic regimen of antibiotics for selective decontamination of the digestive tract (SDD), accompanied by regular microbiological screening of multiple sites [23].

Patient B.S.

This 43-year-old woman in previous good health was admitted to the ITU in septic shock having had a laparotomy for faecal peritonitis following a sigmoid myotomy 7 days previously. She required massive colloid transfusion and inotropic

support to maintain blood pressure and organ perfusion. After 3 days of gradual deterioration following admission to the ITU, repeat laparotomy revealed no localised collection of pus. A rise in core temperature to more than 42°C, increasing cardiovascular instability, and worsening renal function prompted a third laparotomy 6 days after admission. A large perforation in the sigmoid colon was discovered with infected free fluid in the abdomen, from which enterococci, *Escherichia coli, Pseudomonas* spp, and *Serratia* spp were cultured. The defect was oversewn, and the abdomen was irrigated. Her recovery from this operation was stormy with profound hypotension and renal, respiratory, and liver failure. Clinical improvement was obtained by repeated blood product replacement, inotropic infusion, and haemodialysis. Despite maximal supportive treatment, her condition deteriorated inexorably from the 15th day of her admission, and she died after 34 days in the ITU. Repeated blood cultures were negative.

Patient M.C.C.

This 39-year-old man was admitted to the ITU for post-operative care after laparotomy for faecal peritonitis caused by dehiscence of a colonic anastomosis. Three days post-admission, he became floridly septic with hypotension and respiratory and renal impairment. Supportive treatment with volume replacement, inotropic agents, ventilation, and antibiotics led to a rapid improvement in his clinical condition, and he was discharged from the ITU after 10 days. Repeated blood cultures were negative.

Patient M.C.M.

This 32-year-old man was admitted to the ITU in septic shock from pneumococcal septicaemia, with hypotension, respiratory and renal failure, and disseminated intravascular coagulation. He developed severe vasculitis in his lower legs leading to gangrene of both feet. Gradual improvement was achieved with volume replacement, inotropic support, ventilation, and dialysis. An abrupt deterioration with sudden hypoxia and hypotension occurred 14 days post-admission, but re-institution of the previous supportive therapy led to a rapid resolution of the illness. Following bilateral below-knee amputation 21 days postadmission, he was discharged back to the referring hospital.

MATERIALS AND METHODS

Blood Sampling

All patients had blood samples taken on arrival in the ITU and daily thereafter. The blood was taken aseptically after skin preparation with 2% povidone-iodine in alcohol, and serum was removed to pyrogen-free tubes for storage at −20°C for retrospective analysis.

Immunoassay of IgG Anti-LPS Antibodies

Different LPS were complexed with polymyxin-B for coating on polystyrene microplates for enzyme-linked immunosorbant assay (ELISA) of serum IgG antibodies essentially as previously described [20]. Minor modifications were that estimates were made of the different LPS molecular weights based on the reported molecular weight of 3,100 for *Salmonella minnesota* Re595 LPS and a mean

molecular weight of 15,000 for smooth LPS [24], to achieve approximately equimolar concentrations (2.0 μM) of LPS for plate coating. Microplate frames were assembled from 8-well strips (NUNC); each strip was coated with a different LPS. All samples of a given patient's sera were assayed simultaneously as previously described using urease-conjugated anti-human-IgG indicator antibody, and microplate optical density (O.D.) readings were stored directly by a microcomputer for further analysis. Results were processed by microcomputer by plotting each set if IgG anti-LPS O.D. readings net of background (no LPS-polymyxin complexes; strips postcoated with BSA only) on a Hewlett-Packard 7475A plotter.

In all, seven smooth LPS, 22 rough LPS of a range of chemotypes, strains, and species of Gram-negative bacteria, and two lipid A preparations were used in ELISA studies (these will be reported in full together with results of current studies in the future). In this report, we describe the responses to series of LPSs of different sizes from *S. minnesota* smooth and rough mutants, and from a variety of LPSs from smooth strains and complete core rough types of *E. coli* (Table I). These results represent the general trend of changes in IgG antibodies to different LPS chemotypes that were observed in these patients, and are less complex to present than the full set of results. *S. minnesota* LPS and *E. coli* 0111:B4 LPS were purchased from List biologicals (Campbell, CA): other S-LPS were prepared by the method of Westphal et al. [25], and R-LPS were prepared by the method of Galanos et al. [26] as previously described [27].

Endotoxin Assay

Serum endotoxin levels were measured by a microplate modification of a chromogenic *Limulus* amoebocyte lysate (LAL) test kit (Coatest/Endotoxin, Kabi Vitrum Diagnostica UK, Uxbridge, England). Serum samples (0.1 ml) were added to water (0.9 ml) at 75°C and held at 75°C for 5 minutes before cooling rapidly in a 4°C ice/water bath. Two 30 μl volumes of each diluted sample were transferred to flat-bottom microplates. To the first (blank) 90 μl of water was added: to the second

TABLE I. Lipopolysaccharides Used as Antigens in ELISA

Key	Bacteria	Strain	Chemotype
A	*Salmonella minnesota*	R5	Rc
B	*Salmonella minnesota*	R595	Re
C	*Salmonella minnesota*	(ex-R595)	Lipid A
D	*Salmonella minnesota*		S
E	*Salmonella minnesota*	R60	Ra
F	*Escherichia coli* R1	HF 4704	Ra
G	*Escherichia coli* R2	EH 100	Ra
H	*Escherichia coli* R3	F 653	Ra
I	*Escherichia coli* R4	F 2513	Ra
J	*Escherichia coli* K12	W 3110	Ra
K	*Escherichia coli* 06		S
L	*Escherichia coli* 016		S
M	*Escherichia coli* 018		S
N	*Escherichia coli* 086		S
O	*Escherichia coli* 0111		S

(test) 30 μl of LAL was added, followed by 25 min incubation at 37°C, then 60 μl of S2423 chromogenic substrate was added followed by 3 min incubation at 37°C. Acetic acid (60 μl of 50% v/v) was added to both blank and test, and the O.D. was read at 405 nm on a microplate reader relative to reagent blank (30 μl water + 30 μl LAL + 60 μl substrate + 60 μl acetic acid). A standard curve was constructed with *E. coli* 0111:B4 LPS (test kit standard) over a range of 7 doubling dilutions in water from 2.4 endotoxin units (EU), using 30 μl of standard dilution in place of test serum dilution. Results were calculated by subtracting each blank from test for each serum sample, finding the equivalent EU concentration from the standard curve, and multiplying by 10 for the initial serum dilution. Pyrogen-free sterile reagents and equipment were used throughout, and work was performed in a sterile laminar-flow hood.

RESULTS

The daily levels of serum endotoxin and antibodies to different LPS for patients B.S., M.C.C. and M.C.M., together with timing and volume of transfused blood and blood products that may have contributed extraneous antibodies to these patients, are shown in Figures 1–3, respectively.

Patient B.S. showed low but detectable levels of endotoxin in initial samples, which became borderline detectable between days 5 and 11, followed by a rise in endotoxin to a peak of 6.9 EU at day 14, with further small peaks at days 22 and 32. All antibodies to R-LPS and antibody to S-LPS *E. coli* 086 showed considerable fluctuation over days 0–11, most synchronously but some asynchronously, perhaps reflecting some contribution of anti-LPS antibodies by blood products given during this time. All of the above antibodies except anti-*S. minnesota* Ra LPS showed a major depression over days 11 to 14, preceding the major peak of endotoxaemia. All anti-LPS antibodies showed a rise coinciding with the fall of the major endotoxin peak, and most anti-S-LPS and anti-Ra-LPS antibodies (except anti-*E. coli* K12) remained at elevated levels thereafter. Anti-lipid A antibody also remained elevated but showed a wider range of fluctuation. Anti-*S. minnesota* Rc and anti-*E. coli* K12 antibodies showed depressions at days 10–21 and 28–30. Anti-*S. minnesota* Re antibody became profoundly depressed before *each* peak of endotoxaemia and recovered as endotoxin levels fell.

Patient M.C.C. showed high levels of serum endotoxin in the initial sample which fell in the next 3, rose to a second peak on day 5, then fell to a low level with a minor peak on day 9. All anti-LPS antibodies were low to negligible in the initial sample and recovered to intermediate (or high, as for *E. coli* R1, R2, R3, and R4) levels as endotoxin decreased. Most anti-S-LPS and anti-Ra-LPS antibodies showed an interim peak in their recovery on day 5. The recovery in anti-*S. minnesota* Re antibody was latest, beginning at the second peak of endotoxaemia on day 5.

Patient M.C.M. showed no endotoxaemia until day 5, after which endotoxaemia fluctuated then rose to a peak at day 15 and remained elevated. This patient had

Figs. 1–3. Serum endotoxin levels (stippled shading), transfused antibody-containing blood products (columns), and anti-LPS IgG antibodies expressed as net ELISA optical density (O.D.) readings according to the letter key given in Table I, in sequential samples from patients. RBCC = red blood cell concentrate. **Fig. 1:** Patient B.S.; **Fig. 2:** patient M.C.C.; **Fig. 3:** patient M.C.M.

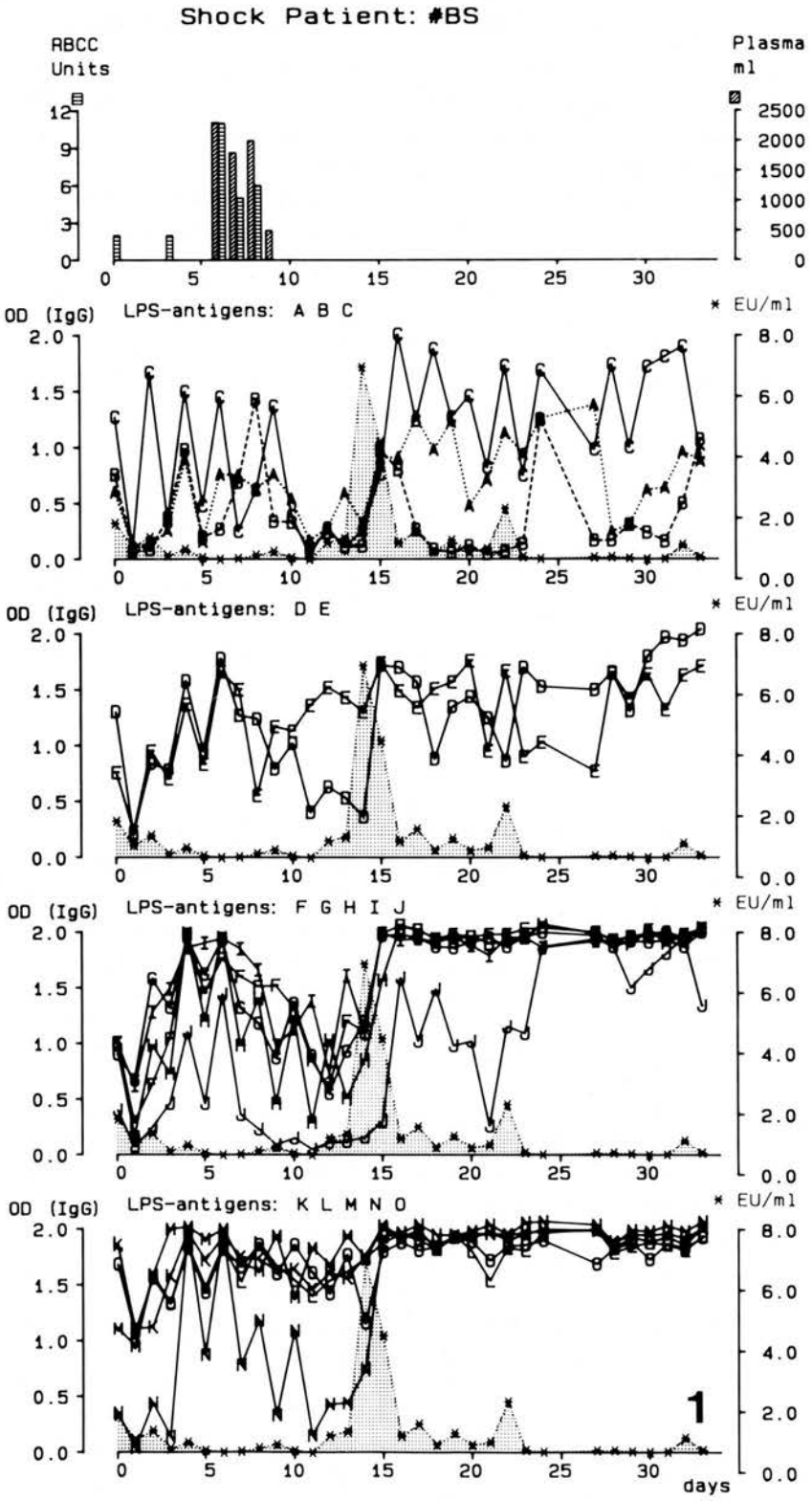
Shock Patient: #BS
RBCC
Units
Plasma
ml
OD (IgG)
LPS-antigens: A B C
* EU/ml
LPS-antigens: D E
LPS-antigens: F G H I J
LPS-antigens: K L M N O
1
days

Fig. 1.

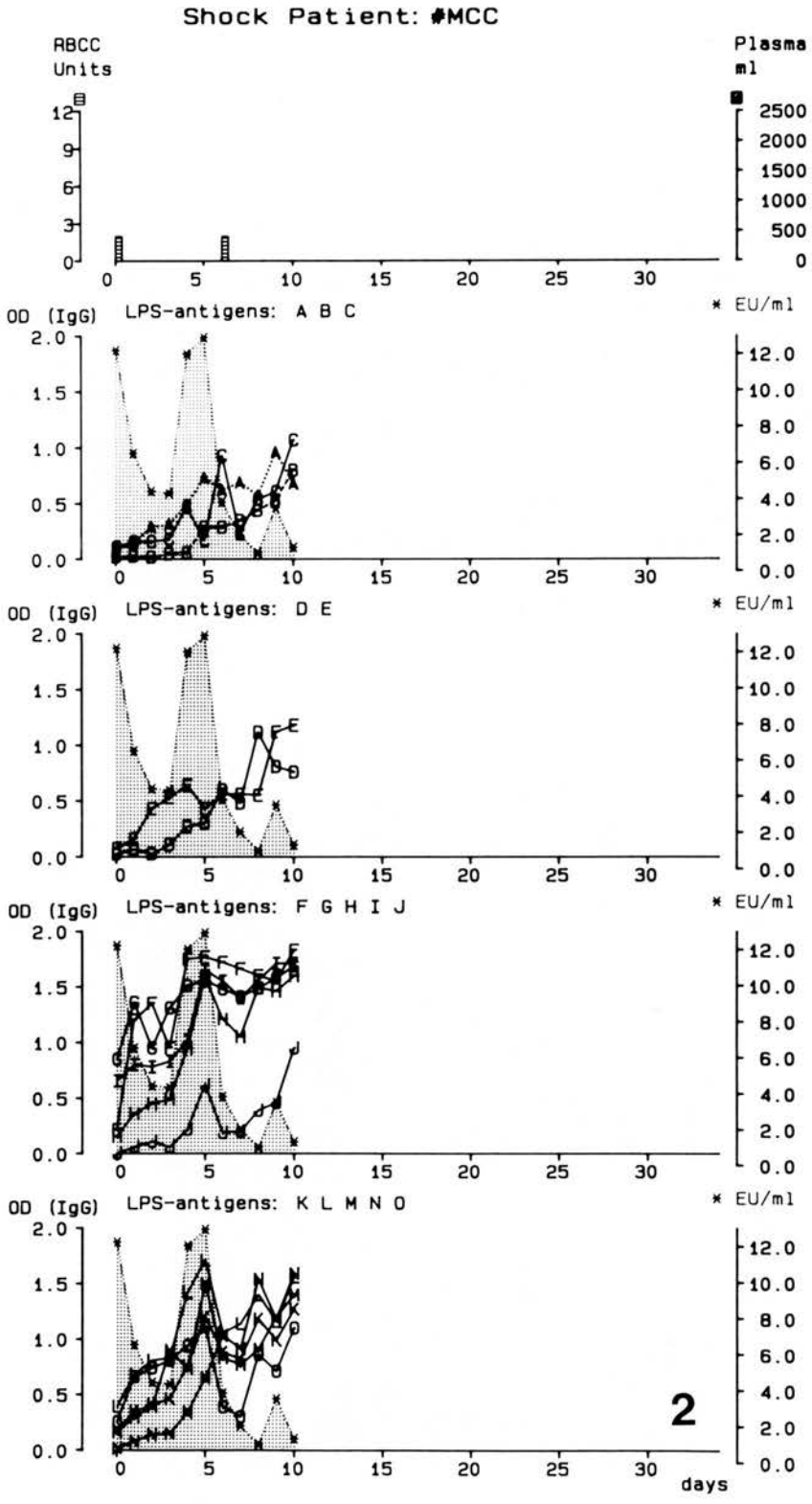
Shock Patient: #MCC
RBCC
Units
Plasma
ml
OD (IgG)
LPS-antigens: A B C
* EU/ml
LPS-antigens: D E
LPS-antigens: F G H I J
LPS-antigens: K L M N O
2
days

Fig. 2.

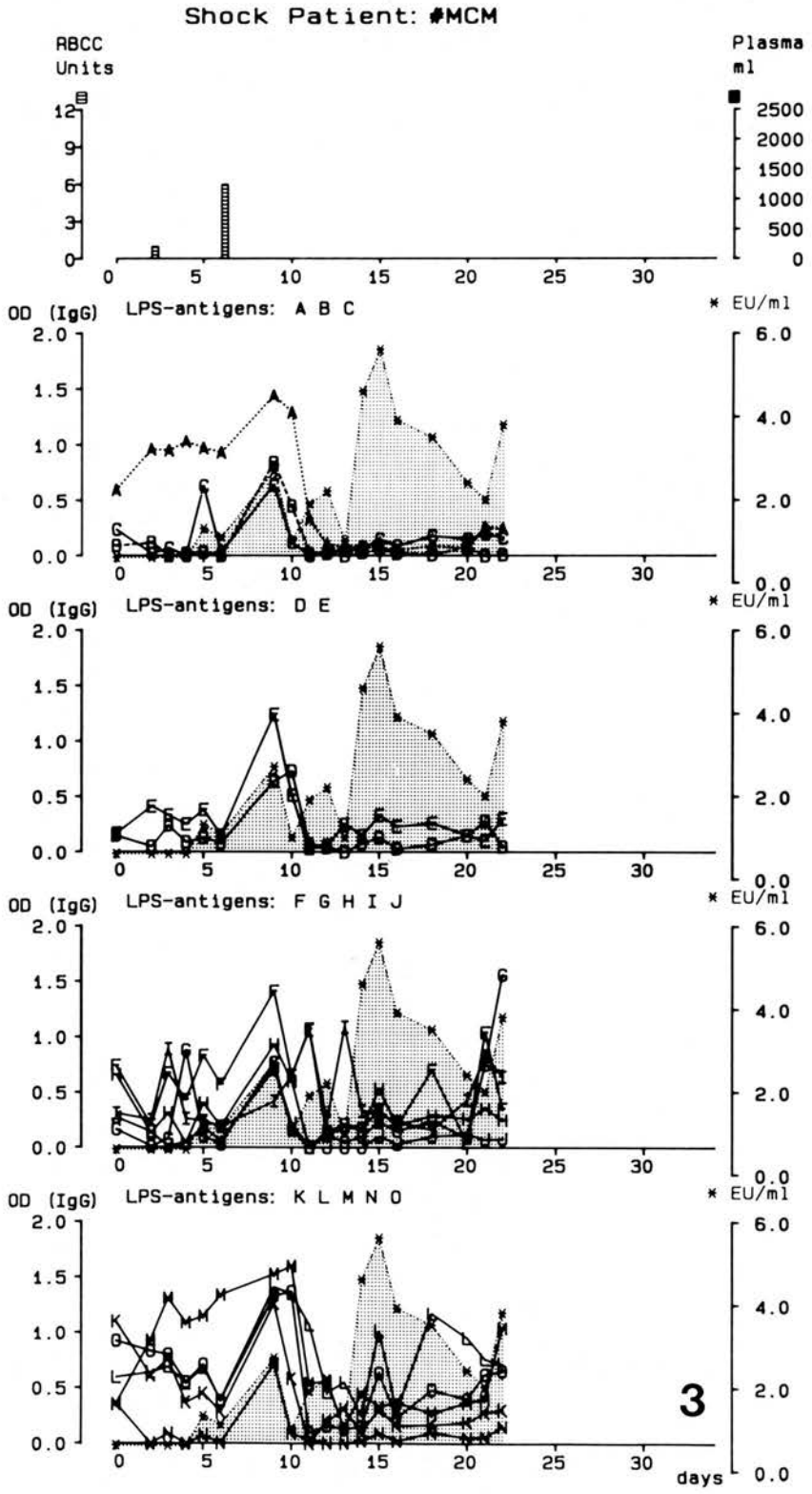
Shock Patient: #MCM
RBCC
Units
Plasma
ml
OD (IgG) LPS-antigens: A B C
* EU/ml
OD (IgG) LPS-antigens: D E
* EU/ml
OD (IgG) LPS-antigens: F G H I J
* EU/ml
OD (IgG) LPS-antigens: K L M N O
* EU/ml
3
days

Fig. 3.

no appreciable levels of anti-R-LPS antibodies except for anti-*S. minnesota* Rc LPS in the early samples, and anti-S-LPS antibodies were generally low. Most antibodies showed a peak after blood transfusion on day 6; this may represent passive antibody. All antibodies, including the anti-*S. minnesota* Rc LPS antibody, became depressed before the peak of endotoxaemia and remained depressed in the presence of elevated endotoxin levels.

DISCUSSION

Despite advances in monitoring and treatment of patients with septic shock, the mortality remains high. Antibiotic treatment is universal in these presumably infected patients, but positive bacteriology to guide treatment is available in only a minority [4,5]. All three patients described here were on continuous SDD prophylactic antibiotics [23] and had regular cultures of sputum, blood, and other secretions; the only positive bacteriology was in patient MCM who had *Streptococcus pneumoniae* grown from the blood. None of the patients showed detectable Gram-negative bacteraemia. All were in septic shock at some stage, diagnosed by standard criteria [28]. Since patients in this ITU on the SDD regimen of continuous antibiotics did not show peaks of endotoxaemia if they were not septic (unpublished observations), it is probable that these patients' endotoxaemias were associated with sepsis rather than intercurrent administration of antibiotics.

Endotoxin is thought to play a central role in septic shock, whether or not Gram-negative bacteria or other organisms can be isolated [7,9,29]. Endotoxaemia may, however, be transient. Initiation by endotoxin of cellular and humoral cascades may result in the typical picture of septic shock without simultaneously detectable levels of endotoxin [7,29]. Recovery from septic shock depends on effective resuscitation during the acute episode, but ultimate survival depends on whether multiple organ failure ensues [30] and the effectiveness of supportive treatment.

The three patients reported illustrate these points. Patient B.S. was in profound septic shock on admission to the ITU and again 6 days later. Endotoxin levels were low or undetectable at those times. On both occasions prompt resuscitation produced clinical improvement. A peak of endotoxaemia on day 15 coincided with worsening renal and hepatic impairment and the development of abnormal neurological signs indicative of marked cerebral injury. She died after 1 month in the ITU owing to chronic and irreversible organ failure. By contrast, patients M.C.C. and M.C.M. survived. Peaks of endotoxaemia on days 5 and 15 coincided with marked clinical deterioration. In both cases resuscitation was effective, and multiple organ dysfunction was only transient.

Whether anti-LPS antibodies played some role in the different clinical outcomes of these three cases cannot be determined. However, it appears that the temporal variations in levels of individual anti-LPS antibodies were related to variations in endotoxaemia, because depressions of certain anti-LPS antibodies occurred immediately before peaks of endotoxaemia, and recovery of these anti-LPS antibody levels preceded falls in circulating endotoxin. Although there was some evidence that administration of blood products could have contributed to rising antibody levels at certain times, our studies have shown that antibodies acquired from infused blood products are detectable very soon after infusion (unpublished). In these cases, we would expect to detect any antibody acquired passively in the next sequential daily

sample. The rising anti-LPS IgG levels during and following endotoxaemia could not be attributed directly to a contribution from administered blood products. Studies of anti-LPS IgG antibodies in healthy blood donors show that different individuals express different patterns of specific antibodies to different parts of the endotoxin inner core [20]. The particular specificity pattern and general levels of antibodies are stable over many months in healthy individuals [31]. In contrast in these patients, wide fluctuations of anti-LPS antibody levels occurred from day to day with certain antibodies, especially those to the inner core of endotoxin, virtually vanishing during endotoxaemia resulting in marked alteration of the relative anti-LPS specificity patterns for this range of LPS antigens. Similar depressions of anti-LPS-CGL antibodies during endotoxaemia have been found by us in a number of other cases of septic shock in shorter series of samples, where the trend of relationships between anti-LPS antibodies and endotoxin levels over an extended time, i.e., whether falling antibody tended to precede rising endotoxaemia and *vice versa*, could not be established because the series were too short (not shown).

The inverse relationship between endotoxin levels and anti-LPS IgG antibodies appears generally true for all of the range of LPS-specific antibodies expressed in patients M.C.C. and M.C.M. Anti-lipid A antibodies tended to reflect the general pattern of variation of the anti-LPS antibodies in these patients, and appeared less sensitive to changes in endotoxin levels than the anti-Re and anti-Rc antibodies when these are expressed. In patient B.S., anti-*S. minnesota*-Re LPS antibodies fluctuated in response to minor changes in endotoxin levels, and antibodies to smaller R-LPS also showed extensive changes, whereas antibodies to most of the range of larger rough LPS (Ra chemotype) were depressed only before the major endotoxin peak on days 14–15. In this case, little variation in anti-S-LPS antibodies was found except for anti-*E. coli* 086 where the pattern of variation resembled that of antibodies to complete core (Ra chemotype): this probably reflects anti-LPS-CGL antibodies bound by this smooth LPS, and not specific anti-086 serotype activity. We have found that a large component of serum IgG antibodies that react with S-LPS can be absorbed by rough Gram-negative bacteria or inhibited by R-LPS in immunoassay [37], and therefore appear to be specific for LPS-CGL, not O-polysaccharide.

We can speculate that the concerted changes in antibodies to a wide range of different LPS, seen especially in patients M.C.C. and M.C.M., might reflect changes in a limited spectrum of IgG anti-LPS antibodies with extensive cross-reactivity. The different patterns of changes in antibodies to different LPS in patient B.S. might indicate a more selective interaction between her endotoxaemia and a subset of her LPS-cross-reactive antibodies, possibly only in the LPS inner core region. Alternatively, in patients M.C.C. and M.C.M. the concerted changes in antibodies with a wide range of anti-LPS reactivities might reflect endotoxaemia caused by a mixture of LPS from different bacterial strains, while the more restricted specificity of the changing anti-LPS antibodies in patient B.S. might reflect a more homogeneous source of endotoxin. In these cases, in vitro absorption studies were not carried out to attempt to identify the range of specificities of the anti-LPS-CGL antibodies in these sera at different times. Definition of the specificities, cross-reactivities, and range of anti-LPS antibodies that change in relation to endotoxaemia in such cases might reveal antibodies whose protective properties should be investigated.

Schedel [32] has reported that in Gram-negative bacteraemia low anti-LPS-CGL

antibodies together with rising or constant endotoxaemia are associated with early mortality. Other studies of anti-LPS-CGL antibodies in Gram-negative bacteraemia, where endotoxin levels were not measured, also indicate a poor prognosis if anti-LPS-CGL antibodies are low [33,34]. Our studies on these three cases of endotoxaemia without detectable Gram-negative bacteraemia appear to indicate that depression of circulating antibodies to the LPS-CGL may reflect consumption of anti-LPS-CGL antibodies by endotoxin, followed by appearance of detectable levels of endotoxin in the peripheral blood. These studies are supported by the observations of Wessels et al. [35] following induction of shock in primates after infusion of *E. coli,* where bacteraemia was short lived, but developing and sustained endotoxaemia was accompanied by a sustained reduction in intrinsic plasma IgG anti-LPS antibodies.

It also appears that the resurgence of certain anti-LPS-CGL antibodies may occur during endotoxaemia and precedes rather than coincides with the disappearance of circulating endotoxin. We may speculate that rising anti-LPS-CGL antibodies may reflect stimulation of secondary humoral responses to endotoxin and that increased output of such antibodies may contribute to the disappearance of circulating endotoxin. While humoral immunity to LPS tends to be viewed as predominantly T-cell independent and refractory to establishment of secondary memory responses, it may be that this only applies to immunity to the repeating polysaccharide units of the O-chain of smooth LPSs. Secondary immunity to cross-reactive components of endotoxin cores, capable of rapid elicitation in response to endotoxin challenge, may be a necessary component of the host defence against endotoxaemia [20]. We have found that immunity to LPS-CGL can be established in rabbits immunised with either rough or smooth Gram-negative bacteria and that rapid-recall anti-LPS-CGL IgG antibody responses can be elicited following subsequent challenge with different strains of Gram-negative bacteria [31]. In one of the above cases (patient B.S.), the endotoxaemia was followed by irreversible multiple organ failure and death. In such cases, supplementation of intrinsic immunity by passively administered antibodies of appropriate anti-LPS-CGL specificity prior to or during endotoxaemia might result in prevention or more rapid resolution of endotoxaemia and a consequent reduction in sustained organ failure and mortality in septic shock. Our current studies, on antibodies from "naturally immune" blood donors and on LPS-cross-reactive monoclonal antibodies, aim to investigate the therapeutic potential of passively administered antibodies for treatment of Gram-negative sepsis.

The LPS occurring naturally in Gram-negative bacteria is of heterogeneous chain length, ranging from smooth forms of various chain lengths to complete unsubstituted core (Ra chemotype). Smaller rough LPS, useful for serological studies, occur in bacterial mutants that are too serum-sensitive to survive in a host, so that the natural serology of anti-LPS antibodies arises as a response to these larger forms of LPS. The serology of Gram-negative LPS is complex, and the precise structures of these LPS have not been fully resolved. The relationship between size and conformation of rough LPS and the effect of this on expression of three-dimensional epitopes is not understood. The effects of purification and presentation for immunoassay may also alter LPS epitope expression. Our novel ELISA, in which LPS is complexed to polymyxin B, appears to counteract some of the effects of LPS presentation in immunoassay. The LPS-polymyxin complexes detect anti-LPS-antibodies not detected by ELISA based on purified LPS alone, and

the complex may mimic LPS structures found when LPS is naturally complexed with bacterial outer membrane proteins or serum proteins, which are the most likely form of presentation of LPS to the host's immune system [20,36,37]. Similar enhancement of detection of anti-LPS antibodies in ELISA can be achieved with other cationic proteins complexed with LPS [37]. Such assays may assist in determining the precise specificities of any anti-LPS-CGL antibodies showing protection against the physiological effects of endotoxins.

A number of reports have tended to emphasize the importance of anti-lipid A antibodies in Gram-negative septicaemia [30,32] or have examined antibodies to individual R-LPS [16,22]. Our studies suggest that cross-reactive IgG antibodies to a variety of epitopes in the endotoxin core are prevalent in normal sera [20,30,35] and can be depressed during endotoxaemia in Gram-negative septic shock. While this report is restricted to three well-documented cases, other recent studies by us of different clinical conditions (to be published) have provided us with evidence that depression of anti-LPS-CGL cross-reactive IgG antibodies during endotoxaemia may be a common phenomenon. In these cases of septic shock, it appears probable that the decrease in serum anti-LPS antibodies represents their consumption, equivalent to in vivo absorption, either by endotoxin or source Gram-negative bacteria derived either from an infective focus or endogenously from the gut [1,8,9].

This study's primary aim was to determine whether IgG anti-LPS antibodies became reduced in septic shock, and to begin to investigate systematically the appropriate specificities of antibodies that might be replaced by passively administered IgG anti-LPS antibody of the same specificity, obtained from plasma of selected blood donors who show high natural levels of such antibodies. Recent studies have suggested that sera from humans or animals deliberately immunized to produce antibodies to the *S. minnesota* Re LPS core are cross-protective against bacteraemia and endotoxaemia when given passively to animals, but that the protection was provided by the IgM component, and not the IgG component, of immune sera [38,39]. Although these reports infer that the pursuit of a suitable gammaglobulin for passive immunotherapy of septic shock from immunized donors may be in vain, it may be that "naturally immune" IgG from selected blood donors may have a different range of anti-LPS specificities and protective properties from "Re-immune" IgG or IgM. However, these reports emphasise the need for further characterization of the properties of anti-LPS antibodies and for determination of whether they offer protection against endotoxic shock. Our study shows that certain IgG antibodies do show changes that appear to be related to episodes of endotoxaemia and supports our continuing investigation of the properties of "naturally immune" blood donor plasma required for selection for preparation of hyperimmune anti-LPS-CGL gammaglobulin for passive immunotherapy of septic shock.

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The experiments described in this paper were performed in adherence to the NIH guidelines for the use of experimental animals.

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IgG Antibodies to Gram-negative endotoxin in human sera. I. Lipopolysaccharide (LPS) cross-reactivity due to antibodies to LPS core

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A series of 700 blood donor sera were screened for IgG antibodies to the core of Gram-negative bacterial endotoxin with a quantitative enzyme-linked immunosorbent-assay (ELISA), based on a cocktail of incomplete-core R-LPS from four different Gram-negative bacterial species, and further serum samples were obtained from donors exhibiting a range of different reactivity for isolation of serum IgG. Analysis of the different IgG samples by ELISA employing a panel of individual LPS from 31 different Gram-negative bacteria covering a range of species, serotypes and R-LPS chemotypes showed that high-titer sera from the screening ELISA expressed IgG with multiple reactivity to LPS in the complex ELISA. We investigated this multiple reactivity in three serum IgGs by inhibition and absorption of isolated serum IgG ELISA reactivity to R-LPS, employing purified LPS and whole bacteria respectively. In two cases the ELISA reactivity appeared to be predominantly attributable to a single antibody component directed to the inner LPS core structure in the lipid A to KDO region. For the third serum IgG, the results suggested that the cross-reactivity may be attributable to more than one specificity-group of cross-reactive antibodies, although still restricted to the LPS inner core structures.

Keywords: IgG, endotoxin, LPS-core, ELISA.

Introduction

The potential of antibodies to Gram-negative bacterial outer membrane lipopolysaccharide (LPS) for passive immunotherapy of Gram-negative bacteraemia and endotoxaemia has attracted many investigators[1,2]. While antibodies to LPS polysaccharide chain (O-serotype specific) can be potently bactericidal[3,4], their clinical usefulness is restricted by the wide variation in LPS O-serotypes[5]. Antibodies to the relatively conserved core-glycolipid region of LPS might show wide cross-reactivity, and might present the best potential for immunotherapy by inhibiting the endotoxic activities of LPS, associated predominantly with the lipid A region of the LPS core-glycolipid[6,7].

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Three prospective sources of cross-reactive anti-LPS antibodies for passive immunotherapy have been cited in the literature: (i) immune plasma for immunized volunteers[6,7]; (ii) plasma of naturally immune blood donors[8–13]; and (iii) monoclonal antibodies raised against the core-glycolipid[3,14–18]. The first two sources have been used with demonstrated clinical benefit. However, immunization with LPS core, e.g. on rough (R) mutant bacteria, can produce a dominant R-LPS specific antibody response instead of cross-reactive anti-LPS responses[20,21]: this problem also arises in the production of monoclonal antibodies. If suitable high-titer donors can be identified then 'natural' anti-LPS antibodies obtained from healthy adults may represent a good source of therapeutic antibodies. Current plasma fractionation techniques best support the production of immunoglobulin G (IgG) antibodies from plasma, and we have therefore focused our studies on this class of antibodies.

The relationship between cross-reactive antibody specificities and clinical efficacy remains to be conclusively established. Neither the structures of the shared epitopes to which cross-protective antibodies are directed nor the means by which they confer protection, either intrinsically or as passive immunotherapy, in different clinical aspects of Gram-negative bacteraemia and septic shock, are fully defined[2]. We have recently demonstrated depression of intrinsic serum IgG antibody levels to structures in the inner LPS core region during endotoxaemia in patients with Gram-negative septic shock, while their antibodies to LPS structures in the outer core or O-polysaccharide region can remain unaltered during these episodes[19]. Thus the passive administration of similar cross-reactive IgG antibodies to LPS inner core during endotoxaemia might mediate some clinical benefit by reducing the level or duration of endotoxaemia and its subsequent clinical effects.

In a previous study we found that IgG antibodies with apparent LPS-cross-reactivity and endotoxin core specificity were common in healthy adult human sera[23]. We now report a study in which we investigated the extent of IgG reactivity to a panel of 31 LPS from different Gram-negative bacteria, encompassing a range of rough chemotypes (R-LPS) and a selection of smooth serotypes (S-LPS), to define the range of specificities to the LPS core that exist in such sera. Random blood donor sera were screened in a quantitative ELISA for levels of IgG antibodies to a cocktail of selected R-LPS. Follow-up sera were obtained from 32 donors selected to show different levels of reactivity in the screening ELISA, and IgG was isolated from sera and evaluated for reactivity to the panel of 31 different LPS by ELISA.

Individuals' sera which showed strong reactivity in the screening ELISA tended to show multiple LPS reactivity to most of the 31 LPS in the panel. To investigate whether the multiple reactivity observed was due to cross-reactive antibody, we attempted to define common structures in the LPS core to which the IgG reacted by absorbing out reactivities from IgG isolated from three different individuals' multiply-reactive sera. Absorptions were made with different *Salmonella minnesota* and *S. typhimurium* rough mutant bacteria with R-LPS of different chain lengths ranging in size from lipid A to complete core. These were employed sequentially in both ascending and descending LPS-size sequence. ELISA inhibition studies were also performed with purified LPS. In an accompanying paper, we report the effects of absorption of anti-LPS core antibodies on serum reactivity with smooth (S) bacteria and LPS[24].

Materials and methods

Lipopolysaccharides

The 31 purified LPS used in ELISA are listed below. The smooth LPS were purified by phenol extraction after the method of Westphal & Jann[25] and the rough LPS were purified by phenol–chloroform–petroleum extraction after the method of Galanos *et al.*[25]. Certain LPS, including lipid A, were purchased from List Biologicals (California, U.S.A.).

The following LPS were prepared in the Dept. of Bacteriology, University of Edinburgh:

S. typhimurium smooth	(O)	*E. coli* R1 (HF4704)	(Ra)
S. typhimurium 1542	(Ra)	*E. coli* R2 (EH 100)	(Ra)
S. typhimurium 119	(Rb)	*E. coli* R3 (F 673)	(Ra)
S. typhimurium 878	(Rc)	*E. coli* R4 (F 2513)	(Ra)
S. typhimurium 1032	(Rd)	*E. coli* C62	(Ra′)
S. typhimurium 1102	(Re)	*E. coli* O6	(O)
		E. coli O16	(O)
P. aeruginosa PAC605	(Rb)	*E. coli* O18K1	(O)
P. aeruginosa 01	(O)	*E. coli* O18 (K1-)	(O)
		E. coli O86a	(O)
K. aerogenes M10b	(Rb)		

The following LPS were purchased (List Biologicals):

S. minnesota smooth	(O)	*E. coli* O111:B4	(O)
S. minnesota R60	(Ra)	*E. coli* K12 mm294	(Ra)
S. minnesota R345	(Rb)	*E. coli* K12 D31m4	(Re)
S. minnesota R5	(Rc)	*E. coli* ex-K12 diphosphoryl-lipid A	
S. minnesota R7	(Rd)		
S. minnesota R595	(Re)		
S. minnesota ex-R595 monophosphoryl-lipid A			

Bacteria

The bacteria used in these studies were as follows:

S. typhimurium 1542	(Ra)	*S. minnesota* R60	(Ra)
S. typhimurium 878	(Rc)	*S. minnesota* R5	(Rc)
S. typhimurium 1102	(Re)	*S. minnesota* R595	(Re)

These were grown in tryptic soy broth (Difco) overnight at 37°C in a shaking incubator then washed in Hank's buffered salt solution (HBSS) (Flow Labs) and finally resuspended at the desired concentration in HBSS.

Lipid A bacteria were produced by acid hydrolysis of *S. minnesota* (R595) and *S. typhimurium* (1102) Re mutants. These were acid hydrolysed after preparation as above by incubating at 100°C in 1% (v/v) acetic acid for 1 h followed by washing in HBSS.

ELISA methods

(a) Quantitative screening ELISA. This assay was developed for screening blood donor sera for recruitment of anti-LPS hyperimmune plasma donors. In brief, the

ELISA employed a cocktail of four incomplete core R-LPS from different species of Gram-negative bacteria (*E. coli, P. aeruginosa, K. aerogenes* and *S. typhimurium*) complexed with polymyxin as below and mixed in equimolar proportions for coating on polystyrene microplates. Test serum results were compared with a dilution curve of a reference serum known to contain high-titer LPS-cross-reactive IgG antibodies. Samples were tested at a 1:400 dilution. All tests were performed in triplicate, with a single control antigen-negative well (see below). The ELISA was developed by urease-conjugated sheep anti-human IgG antibody followed by addition of urease substrate (Commonwealth Serum Laboratories, England), and results were expressed as a percent of the reference serum reactivity, calculated from the standard curve by processing microplate reader (Titertek Multiscan) optical density (OD 585 nm) readings fed directly into a microcomputer and using computer programmes developed by us.

(b) Complex LPS panel ELISA. The LPS-polymyxin ELISA was performed essentially as described previously[26] except that the LPS-polymyxin complexes were formed by reacting LPS and polymyxin together at 0·1 mM and 0·2 mM respectively, using the molecular weight (MW) of polymyxin and the approximate MWs of the various LPS as reported by Morrison & Jacobs[27] as a guide for our own calculations of the LPS MW. LPS and LPS-polymyxin complexes were sonicated for 30 s. Complexes were coated on to polystyrene microplate 8-well strips (Nunc 'polysorb'). Coated strips were stored at −40°C prior to use, and the complex ELISA assay panel was constructed by assembling the 31 different coated strips across three ELISA plate frames for each complete panel set. All solutions used in the ELISA procedure were made up with pyrogen-free water containing 0·05% sodium azide.

Samples were measured in ELISA at a 1:100 dilution in duplicate against test antigen, with a single negative-control well for each sample, post-coated with bovine serum albumin only, as previously described[26]. Except where stated samples were reacted simultaneously against the full panel of 31 LPS listed, and results were processed and stored by microcomputer. Results are expressed as or derived from mean OD minus negative control OD.

Purification of IgG from sera

IgG was purified from serum by ammonium sulphate precipitation followed by DEAE cellulose column chromatography. Solid ammonium sulphate was added to serum to 50% saturation, and dissolved by stirring. The resulting precipitate was collected by centrifugation at 10 000 *g* for 60 min. The pellet was resuspended initially in a small volume of 10 mM potassium phosphate at pH 6·8 then adjusted to the original serum volume. This solution was then reprecipitated, centrifuged and resuspended as before. The salt was removed by ultrafiltration by vacuum dialysis (Sartorius Collodion, Membrane size 12400 MW cut-off) against three volumes of 10 mM potassium phosphate and readjusted to the original volume.

Purification of the IgG from this solution was performed on a DEAE cellulose column (Whatman DE52) equilibrated to pH 6·8 of the 10 mM potassium phosphate buffer. The IgG peak was monitored by measuring column effluent absorbance at 280 nm. The remaining immunoglobulins and serum proteins on the column were eluted by increasing the ionic strength of the buffer by the addition of 1·4 M NaCl. The IgG peak was reconcentrated to the original volume of each serum by vacuum dialysis, to restore the

approximate physiologic concentration of IgG. Purity of IgG preparations was measured by immunoelectrophoresis, and concentration determined by laser nephelometry.

Inhibition and serial absorption protocols

Inhibition of ELISA by LPS. IgG, diluted 1:100 in ELISA dilution buffer, was mixed with a polymyxin-LPS-complex (dilution as specified and prepared as described above) and preincubated at 37°C for 1 h with occasional shaking before adding to the ELISA. The mixture was treated in ELISA in duplicate against *S. minnesota* R5 (Rc) LPS (and negative control).

Serial absorption of IgG on whole bacteria. IgG were absorbed serially by bacteria in ascending and descending order of LPS core size in the following series:

(1)	*Descending series*	(2)	*Ascending series*
(A)	*S. minnesota* R60 (Ra)	(A)	*S. minnesota* lipid A
(B)	*S. minnesota* R5 (Rc)	(B)	*S. minnesota* R595 (Re)
(C)	*S. minnesota* R595 (Re)	(C)	*S. minnesota* R5 (Rc)
(D)	*S. minnesota* lipid A	(D)	*S. minnesota* R60 (RA)
(3)	*Descending series*	(4)	*Ascending series*
(A)	*S. typhimurium* 1542 (Ra)	(A)	*S. typhimurium lipid* A
(B)	*S. typhimurium* 878 (Rc)	(B)	*S. typhimurium* 1102 (Re)
(C)	*S. typhimurium* 1102 (Re)	(C)	*S. typhimurium* 878 (Rc)
(D)	*S. typhimurium* lipid A	(D)	*S. typhimurium* 1542 (Ra)

Serum IgG solutions (1 ml), diluted 1: 10 in HBSS containing 0·05% sodium azide, were added to Minisorb tubes (Nunc) containing sedimented washed bacteria at 10^7 cells ml^{-1}. Bacteria were resuspended in the IgG solutions and incubated for 15 min at room temperature. The bacteria were then centrifuged at 1500 ***g*** for 5 min. The supernatants were removed to fresh tubes of sedimented bacteria and the process repeated. This step was repeated three times for each absorbing bacteria (sufficient to maximally absorb antibody on each bacteria), and an aliquot of IgG solutions was removed after absorption by each strain of bacteria in the serial sequence, for ELISA tests.

The reactivity in ELISA of IgG, further diluted in the ELISA dilution buffer[26] to give a final dilution of 1: 100, was measured in duplicate with a background control well after each step in each series against a panel of LPS (*S. minnesota* O (smooth), Ra, Rc, Re, lipid A; *S. typhimurium* O (smooth), Ra, Rc and Re).

Results

Reactivity of IgG isolated from sera of blood donors to a panel of 31 different LPS in ELISA

Randomly selected blood donor sera (700) were evaluated by the R-LPS cocktail quantitative ELISA. Figure 1 shows the distribution of the results. Donors selected across this range of distributions, from high through intermediate to low, were approached for further samples of sera. A total of 32 volunteers each donated a further

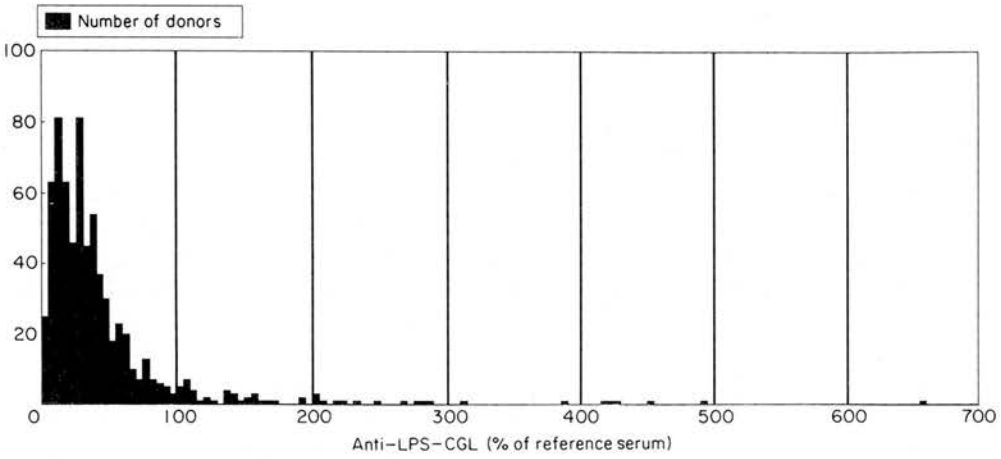

Figure 1. The distribution of IgG antibodies to a cocktail of R-LPS from four different Gram-negative bacterial species (LPS core glycolipids, LPS-CGL) in sera from 700 randomly-selected healthy adults (Blood donors) measured by a quantitative ELISA. Results are given as the numbers of donors with different anti-LPS IgG antibody levels (levels are expressed as a percentage of ELISA reactivity of a high-titre anti-LPS-core cross-reactive serum, used as the reference serum in the quantitative assay).

50 ml blood sample from which serum IgG was purified for study in isolation from other immunoglobulin classes.

Purified IgG solutions from the 32 different blood donors' sera were evaluated in ELISA against 31 different LPS. The results shown in Figure 2 represent typical reaction patterns found in this ELISA, namely multiple reactivity (MR), partial reactivity (PR), and weak reactivity (WR). IgG MR-1, MR-2 and MR-3 [Fig. 2(a)] show strong reactions with most or all of the 31 LPS antigens, and were all derived from the high region of the distribution (>200%, Fig. 1). IgG PR-1, PR-2 and PR-3 [Fig. 2(b)] show weak reactions with many of the LPS antigens but also a small number of stronger reactions to some of the LPS antigens, and were derived from the mid-range population (30–60%, Fig. 1). IgG WR-1, WR-2 and WR-3 [Fig. 2(c)] show only weak reactions or no reaction, and were derived from the low region of the population (<10%, Fig. 1). No IgG failed to show some reactivity with at least some of the rough LPS antigens at the dilution tested.

Sequential absorptions by whole bacteria of ELISA reactivity of purified serum IgG to a range of Salmonella LPS chemotypes

Three multiply-reactive IgG preparations (MR-1, MR-2 and MR-3) were absorbed sequentially with whole bacteria in each of the patterns listed in the methods. The residual reactivity in the ELISA of each IgG after each stage of each series of absorption was measured against purified LPS from *S. typhimurium* wild-type, 1542 (Ra), 878 (Rc), 1102 (Re), *S. minnesota* wild-type, R60 (Ra), R5 (Rc), R595 (Re), and lipid A. The results, shown in Figures 3–5, show the percentage residual ELISA reactivity after each absorption.

The results of these absorbance studies reveal a number of complexities which will be discussed. However, with regard to the inner core regions of Salmonella LPS, it appeared that the specificities of antibodies in IgG preparations MR-2 and MR-3 were

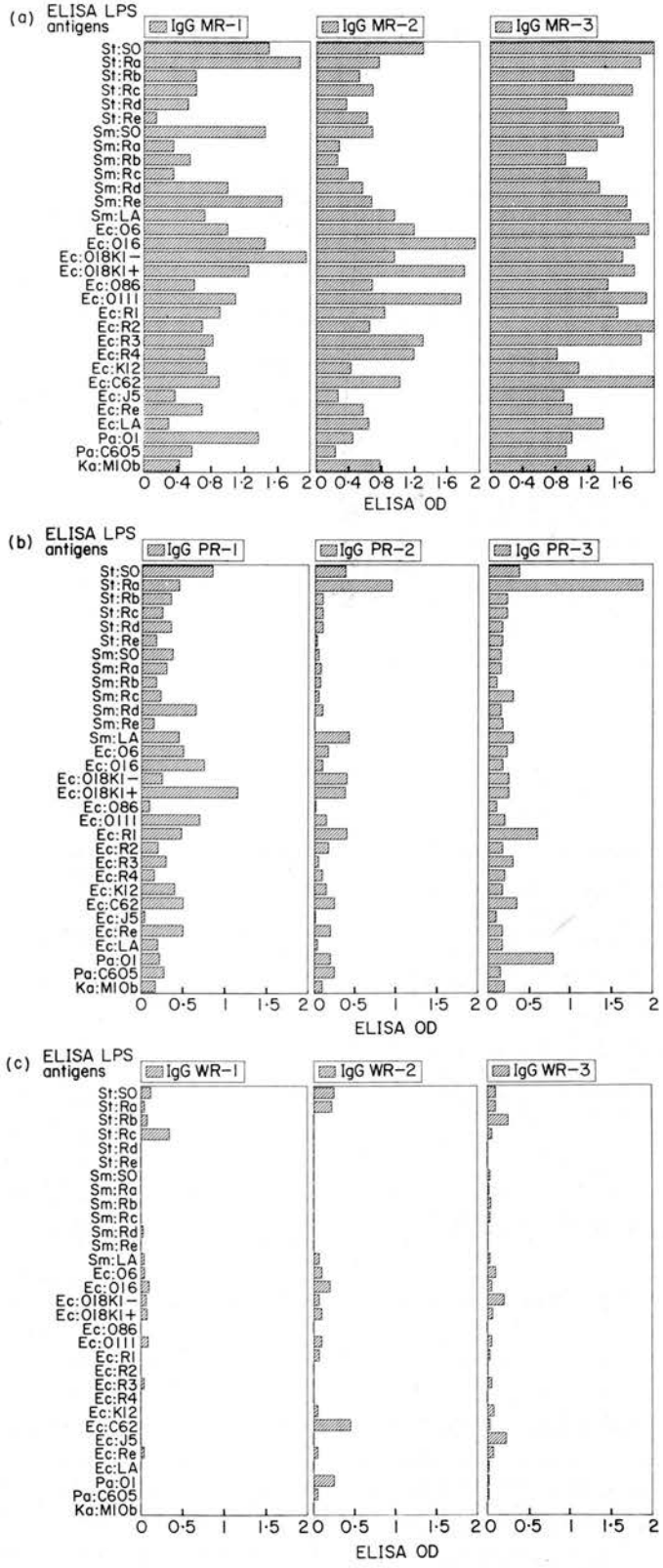

Figure 2. The ELISA reactions to 31 different individual LPS of IgG isolated from sera of different individuals with (a) multiple anti-LPS reactivity, (b) partial anti-LPS reactivity, and (c) weak anti-LPS reactivity. The bars represent net ELISA OD readings (mean of duplicates, minus background) for each LPS. St: *Salmonella typhimurium*, Sm: *Salmonella minnesota*, Ec: *E. coli*, Pa: *Pseudomonas aeruginosa*, Ka: Klebsiella aerogenes. Unless strain is prefixed by 'O', the LPS is rough.

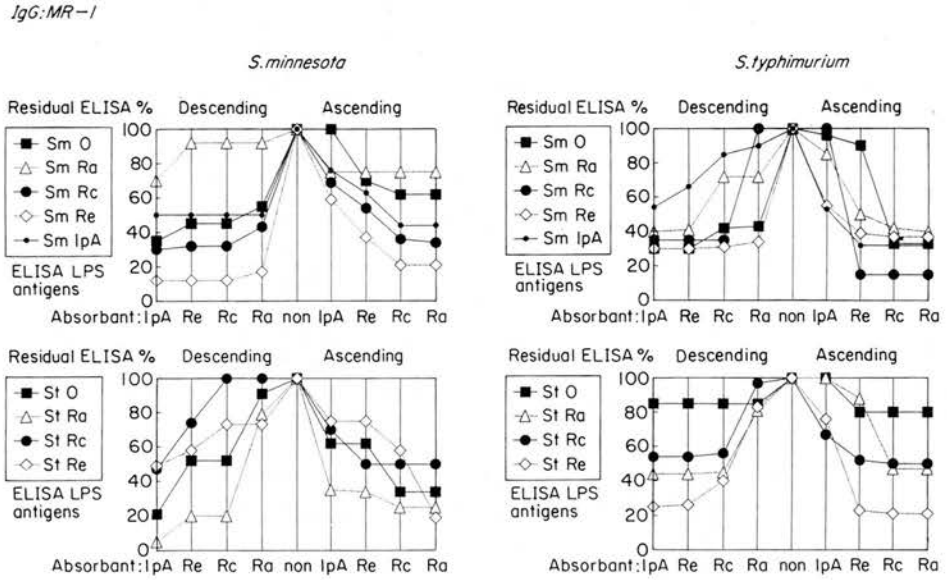

Figure 3.

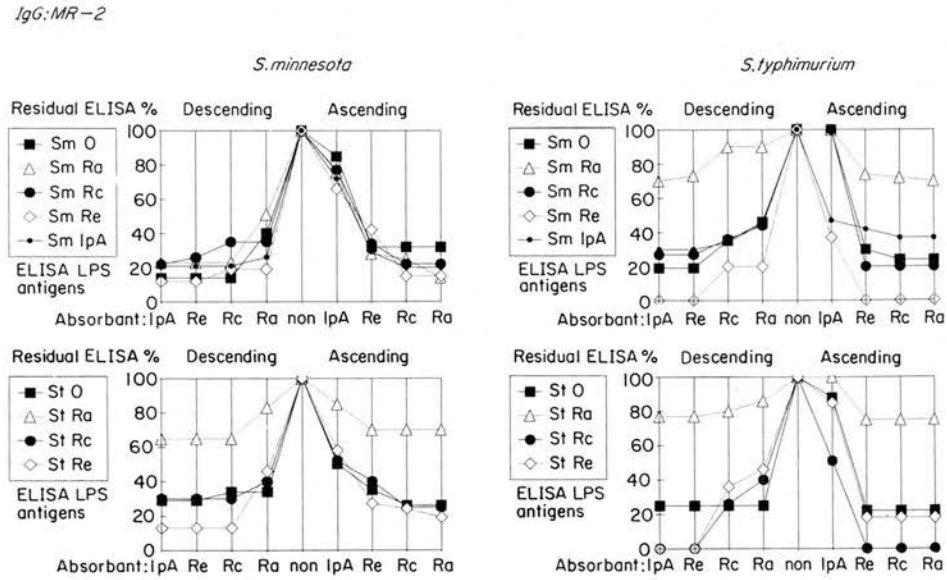

Figure 4.

similar and largely absorbed out on Re or lipid A bacteria, while IgG preparation MR-1 appeared to be only partially absorbed on Re and lipid A bacteria and required Rc bacteria for more complete absorption. A simple interpretation is that MR-1 contains antibodies to both the lipid A/KDO region and heptose region of the LPS core while MR-2 and MR-3 contain predominantly antibodies only to the lipid A/KDO region.

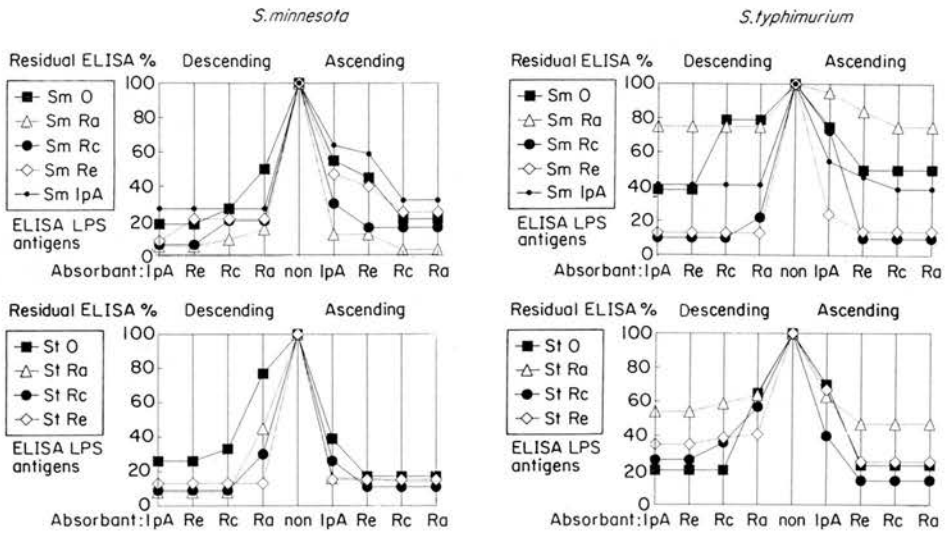

Figure 5.

Figures 3–5. Serial absorbtion of different serum IgG preparations with rough *S. typhimurium* or *S. minnesota* bacteria with different size LPS, which were used in sequential steps in both ascending and descending LPS size to absorb IgGs. Residual ELISA reactivities to *S. typhimurium* or *S. minnesota* S-LPS and R-LPS at each stage of absorption are expressed as a percentage of the reactivity in unabsorbed IgG.

Comparing IgGs MR-2 and MR-3 (Figures 4, 5), the 'lipid A' acid-hydrolysed bacteria absorbs antibodies from MR-3 better than from MR-2 which requires in addition Re bacteria for more complete absorption. Thus, MR-3 may contain antibodies which may be predominantly lipid A region specific while antibodies in MR-2 may be more KDO-region specific.

Other features which were noted were as follows.

(i) Maximal absorption of antibodies binding to Ra and smooth (O) LPS was achieved only for MR-2 and MR-3 anti-*S. minnesota* Ra and O LPS, on both *S. minnesota* and *S. typhimurium* bacteria expressing LPS of most sizes (Figures 4, 5). In contrast, the same IgG preparations expressed antibodies to *S. typhimurium* Ra LPS which were not readily absorbed even on *S. typhimurium* Ra bacteria.

(ii) In IgG MR-3 (Figure 5), maximal absorption of antibodies to *S. typhimurium* LPS is achieved with lipid A and Re *S. minnesota* bacteria whereas antibodies to *S. minnesota* LPS require in addition the Rc bacteria from the *S. minnesota* series. MR-3 antibodies to *S. minnesota* Ra were poorly absorbed by *S. typhimurium* LPS of any size.

(iii) In IgG MR-1 antibodies to *S. minnesota* (Figure 3), anti-Ra and anti-lipid A were absorbed least while anti-Re and anti-Rc were absorbed best, on either *S. minnesota* or *S. typhimurium* LPS. In contrast, in the antibodies to *S. typhimurium* LPS, anti-Ra was absorbed better than anti-Rc, and the Rc antibodies were not well absorbed until Re LPS was used in the *S. minnesota* series.

(iv) In general, there was little difference between ascending and descending sequences in the final ELISA reactivity of an absorbed IgG preparation for a given LPS after a complete absorption sequence.

Inhibition by purified LPS (as LPS-polymyxin complexes) of ELISA reactivity of purified serum IgG to S. Minnesota *Rc LPS*

The three multiply-reactive IgG preparations were also studied by inhibition of their ELISA reactivity to *S. minnesota* R5 (Rc) LPS, which we have previously shown to be the smallest LPS chemotype with which antibodies in most human sera commonly react in an IgG ELISA[23]. Inhibitions were carried out with LPS complexed with polymyxin, which we have shown to be a more efficient ELISA inhibitor than purified LPS alone[28]. Maximal inhibition of IgG preparations MR-1, MR-2 and MR-3 (Figure 6) was usually observed at the 1:40 dilution of the stock 0·1 mM LPS-polymyxin complex and fell with dilution. For MR-1, the greatest inhibition was caused by *S. minnesota* R5 (Rc) LPS.

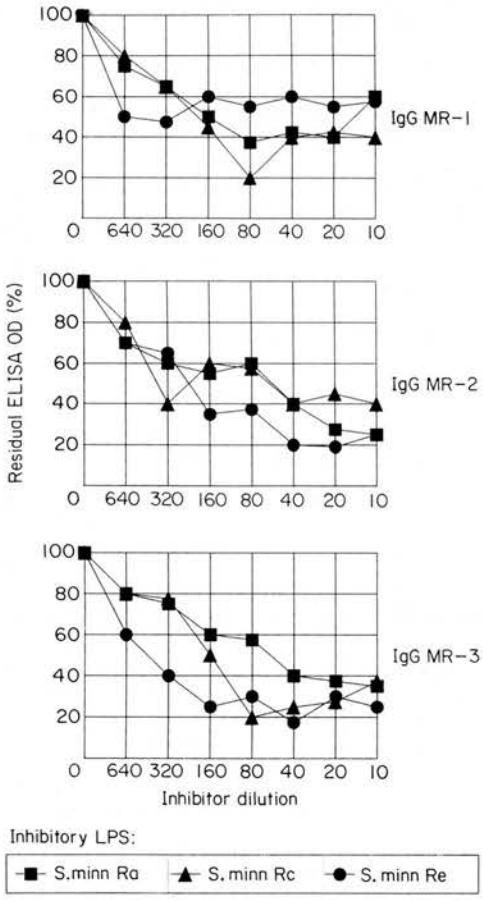

Figure 6. Inhibition of ELISA reactivity of different IgG preparations to *S. minnesota* R5 (Rc) after preincubation with different size R-LPS complexed with polymyxin. Residual ELISA reactivity in the presence of LPS is expressed as a percent of reactivity with no LPS. LPS inhibitor dilutions are from an original concentration of 0·1 mM.

Inhibition by *S. minnesota* 595 (Re) LPS reached its maximum at the two lowest concentrations of inhibitor tested, and was then apparently saturated with no further inhibition occurring at higher concentrations. Inhibition of MR-1 by R60 (Ra) LPS increased less steeply with increasing concentration than for Rc LPS, but achieved a similar degree of maximal inhibition at the highest concentrations tested. For MR-2 and MR-3, the inhibition curves were similar for all three LPS chemotypes, with inhibition increasing as the concentration of each LPS complex increased. Each LPS-complex reached different degrees of maximum inhibition.

Discussion

The healthy adult population displays a wide range of levels of antibodies to LPS core (Figure 1), and it may be that the individuals who can be identified with high levels of such antibodies could provide a source of plasma for preparation of a therapeutic anti-endotoxin gammaglobulin. Purified IgG from the 32 sera selected from high, medium and low positions in this distribution analysed in ELISA revealed a number of features. Amongst this group of IgGs there were greatly contrasting ranges of reactivities. We found some IgGs which had strong multiple reactivity with all or most LPS in the panel, e.g. IgG MR-1, MR-2 and MR-3 [Figure 2(a)]. The majority of IgGs investigated reacted strongly with only a minority of LPS and weakly with most other LPS, e.g. IgG PR-1, PR-2 and PR-3 [Figure 2(b)]. A third minor group of IgGs showed only weak or negligible reactivities to these LPS under the ELISA conditions used, e.g. IgG WR-1, WR-2 and WR-3 [Figure 2(c)]. No IgG lacked reactivity with at least some of the rough LPS antigens in this ELISA. Under more sensitive assay conditions, some human serum IgG antibody reactivity can be detected to most LPS from different Gram-negative bacterial chemotypes and strains (not shown). This is in agreement with previous reports of antibodies to LPS, which were found to be widespread and common in the adult population[13].

Although these IgGs prepared from individual donors' sera may contain antibodies with a range of anti-LPS specificities, it appears that they contain a major component which is LPS cross-reactive. Although it is difficult to define individual specificities in polyclonal IgG we hoped to identify the region in the LPS core to which the antibodies were directed. Three serum IgGs identified as having strong LPS multiple reactivity by ELISA, MR-1, MR-2 and MR-3 [Figure 2(a)], were evaluated in inhibition and absorption studies to confirm the presence of LPS cross-reactive antibodies with specificity for LPS core.

Considering only the absorption of anti-*S. minnesota* LPS responses by different R-mutants of *S. minnesota* (Figures 3–5) and inhibition of anti-*S. minnesota* Rc LPS responses by *S. minnesota* LPS (Figure 6), it appears that IgG MR-2 and MR-3 have a single predominant group of LPS-core cross-reactive antibodies directed to the deepest core lipid A and/or KDO region, while IgG MR-1 has both this type of antibody and another group of antibodies with reactivity beyond the Re-LPS chemotype size, probably to the heptose region expressed on Rc-LPS.

When we consider the differences in absorptions and anti-LPS reactivities between *S. minnesota* and *S. typhimurium*, it was found that while the results support the above interpretation of the predominant anti-LPS antibody groups in these IgG preparations, there are apparent differences between the 'equivalent' R-chemotype LPS of these strains of Salmonella. In IgG MR-2 this difference is expressed to the outer-core region

as differences in Ra serology, where different *S. minnesota* R-LPS, including Ra-LPS, do not absorb antibodies to *S. typhimurium* Ra-LPS, and vice versa. In this case, the anti-*S. typhimurium* Ra LPS antibodies are not well absorbed even by *S. typhimurium* Ra LPS, while antibodies to both *S. typhimurium* and *S. minnesota* smooth LPS are absorbed on either species Re LPS.

Complete (Ra) core LPS is co-expressed with S-LPS in Gram-negative bacteria which may be encountered by the host, and probably stimulates LPS core 'type' specific antibody responses of limited cross-reactivity. Such antibodies may not be depressed during endotoxaemia in septic shock while antibodies to the LPS inner core are depressed[19]. Since it is difficult to distinguish different antibody activities in polyclonal sera in assays such as Western blotting, we cannot tell at this stage whether these LPS outer-core type-restricted antibodies react only with the Ra LPS component from smooth bacteria or whether they react with complete S-LPS expressing O-polysaccharide chains. Evidence from our studies with monoclonal anti-LPS antibodies (unpublished) suggests that both R-LPS restricted and S-LPS cross-reactive Ra-specific antibodies can arise.

The failure of *S. typhimurium* Ra bacteria to absorb the antibody to *S. typhimurium* Ra LPS appears to indicate that the antibody binding site is better exposed under the conditions of the ELISA than at the absorbing bacterial surface. This also appears to operate for antibodies to *S. minnesota* Ra LPS in MR-1 and MR-3 IgGs. These results appear to indicate differences between these Salmonella species LPS in the outer core region. It was also noted that antibodies to *S. minnesota* lipid A were incompletely absorbed: this may also be due to better expression of antigen under ELISA conditions than on absorbing bacteria. In each case it may be that these natural antibodies from human sera may arise after exposure to endotoxin rather than complete bacteria, since they are poorly absorbed on bacteria but react well in LPS-polymyxin ELISA.

Other studies by us of inhibition of ELISA by LPS have been described in detail elsewhere[28], where it was found that purified LPS was a poor inhibitor of anti-LPS antibodies in this ELISA, whereas purified LPS complexed with polymyxin is a more efficient inhibitor of this ELISA. Crude LPS still complexed with bacterial outer-membrane proteins, and purified LPS complexed with different polycations, including polymyxin, or complexed with serum high-density lipoprotein, are also better inhibitors of this ELISA than purified LPS (manuscript in preparation). The experiments in this inhibition study were therefore performed using LPS-polymyxin complexes. This phenomenon appears to be related to observations made in the development of this LPS-polymyxin ELISA technique, where the LPS appears more accessible to 'natural' antibodies in human sera in the complexed form than in a completely free state[23]. LPS complexed to polymyxin may allow the LPS to mimic its natural conformation where it is complexed to protein in the bacterial outer membrane[29,30] or complexed to various host plasma constituents or cells[31–34].

Although it may not be valid to draw general conclusions from an analysis of such a small number of sera, the results support earlier observations that sera contain predominantly one of two kinds of cross-reactive families of anti-LPS-core antibodies, (i) reactive with both Re-LPS and Rc-LPS ('anti-lipid A/KDO') or (ii) with Rc-LPS but not Re-LPS ('anti-heptose')[23]. The cross-reactive element of IgG MR-2 and MR-3 has been shown to consist primarily of the 'anti-lipid A/KDO' family of antibodies. The identification of two major antibody specificities in IgG MR-1 is not in agreement with earlier observations of apparent mutual exclusivity of these families of antibodies.

However, since this study was conducted on three highly selected sera with high-titer anti-LPS cross reactive antibodies, from the high end of the population distribution (Figure 1), this serum may be exceptional.

We have established that selected sera with high-titer natural IgG antibodies specific for LPS inner core and cross-reactive for Salmonella LPS can be obtained from the adult population. However, it is important to establish the reactivities of such sera with smooth Gram-negative bacteria commonly encountered in bacteraemia leading to septic shock, in particular *Escherichia coli* species. Studies of the interactions of natural LPS-core cross-reactive antibodies are reported in the accompanying paper[24].

Acknowledgement

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A comparison of immunoblotting, flow cytometry and ELISA to monitor the binding of anti-lipopolysaccharide monoclonal antibodies

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This study was designed to assess the use of flow cytometry to observe the binding, under physiological conditions, of anti-lipopolysaccharide (LPS) monoclonal antibodies (mAbs) to whole bacteria, and to compare this with the more conventional whole cell ELISA and immunoblotting techniques. The bacteria consisted of two clinical isolates of *E. coli* 018:K1 and 06:K5 and two isogenic mutants of the 018 parent: a non-capsulate (018:K$^-$) and a rough mutant (018rf). Two cross-reactive anti-core mAbs and one 018 0-antigen-specific mAb were used. ELISA and flow cytometry showed that capsule and O-polysaccharide influenced the binding of mAbs to the bacteria, whilst the latter technique demonstrated that sub-populations existed. Immunoblotting showed the two anti-core mAbs to be different, one bound only to core which was not substituted with O-antigen, whilst the other bound both to substituted and unsubstituted core. This comparison for monitoring the binding of anti-LPS mAbs demonstrates the potential use of flow cytometry in bacterial cell surface research, and complements results obtained by ELISA and immunoblotting.

Key words: Flow cytometry; ELISA; Immunoblotting; Lipopolysaccharide; *E. coli*; Monoclonal antibody

Introduction

Lipolysaccharide (LPS) or endotoxin is a major constituent of the outer membrane of all Gram-negative bacteria and is known to be responsible for the range of pathophysiological features of endotoxic shock. In general, LPS consists of three regions: the outer O-polysaccharide – the composition of which varies with the serotype of the organism, the core oligosaccharide – which is less variable, especially in the inner part, and the inner lipid A – a highly conserved structure, responsible for the toxicity and many of the biological activities of LPS (Rietschel et al., 1984).

Limitations of existing therapeutic agents for endotoxic shock have led to active investigation of protective antibodies. It is thought that antibodies directed against the conserved elements of the core oligosaccharide and lipid A region of LPS may be cross-reactive and possess anti-endotoxic properties (Chedid et al., 1968). An area of great debate is whether antibodies can bind to these inner regions of LPS in its natural states either on the bacterial cell surface or bound to serum components in the circulation (Pollack et al., 1989).

Flow cytometry has been used extensively to analyse eukaryotic cell populations (Melamed et

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al., 1979; Muirhead et al., 1985), although its application to bacteria has remained limited. This study was undertaken to assess the use of flow cytometry for monitoring the binding of different anti-LPS monoclonal antibodies (mAbs) to whole bacteria, and to compare this with the more conventional techniques of enzyme-linked immunosorbent assay (ELISA) and immunoblotting.

Materials and methods

Bacteria and culture conditions

Four strains of *Escherichia coli* were kindly supplied by Dr. A.S. Cross, Walter Reed Institute for Army Research, Washington, DC, U.S.A. They consisted of two clinical isolates (018:K1 and 06:K5 serotypes) together with two isogenic mutants from the 018 parents: a non-capsulate mutant (018:K$^-$) and a rough mutant (018rf). Cultures were grown in 100 ml filter-sterilised nutrient broth (Gibco) in 250 ml conical flasks at 37 °C for 16 h in an orbital incubator. Cells were harvested and washed twice in phosphate-buffered saline, pH 7.4 (PBS). Total counts were made in a haemocytometer (Thoma ruling).

Monoclonal antibodies

Monoclonal antibodies (mAbs) were prepared by fusing spleen cells from immune BALB/c mice with NSO myeloma cells by standard techniques (Kipps and Hertzenberg, 1986). The reactivity of the mAbs was determined by an LPS-polymyxin ELISA method as described by Scott and Barclay (1987). The 018-specific mAb was screened by its binding to 018 LPS, while a cocktail of four rough LPS was used as a primary screen for the anti-core mAbs, followed by a broad secondary screen of both rough and smooth LPS. Full details of the immunisation of mice, selection of hybridomas and characterisation of the mAbs are to be published elsewhere. Three mAbs with known specificities in LPS-polymyxin ELISA were selected for this study: mAb 0-1 (specific for 018 O-antigen), and mAbs C-1 and C-2 (both cross-reactive with core epitopes). Supernatant fluids of hybridoma cell cultures grown in RPMI 1640 supplemented with 5% foetal calf serum in 150 cm^2 flasks were used throughout. Cell cultures were grown to maximum cell density and harvested at 50% cell viability. These three hybridomas gave yields of approximately 50 μg/ml.

Preparation of LPS

LPS was prepared from whole washed bacteria by the Proteinase K method of Hitchcock and Brown (1983). The method is described in detail by Hancock and Poxton (1988). Briefly, bacteria grown overnight were washed twice in PBS and adjusted to an A_{525} between 0.5 and 0.6. After centrifugation, the pellet from 1.5 ml of the bacterial suspension was suspended in 50 μl of sample buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue in 1 M Tris-HCl pH 6.8) and heated for 10 min at 100 °C. Proteinase K (25 μg in 10 μl sample buffer: protease Type X1, Sigma) was added, and the mixture incubated at 60 °C for 60 min.

SDS-PAGE

SDS-PAGE was performed on 14% acrylamide slab gels with the Laemmli buffer system (Laemmli, 1970). Samples (10 μl for silver stain, or 20 μl for immunoblotting) of the proteinase K LPS extracts were loaded on to the gels. The LPS separating gels were stained with silver by a method developed by Tsai and Frasch (1982), modified by Hancock and Poxton (1988). Briefly, gels were fixed overnight in 200 ml of 25% propan-2-ol, 7% acetic acid followed by oxidation in freshly prepared periodic acid. After frequent washing over 4 h in distilled water, fresh ammoniacal silver nitrate solution (1.4 ml of ammonia solution, 21 ml of 0.36% NaOH, 4 ml of 19.4% $AgNO_3$ and distilled water to 100 ml) was added for 15 min. Following at least four washes in distilled water over 40 min, gels were transferred to fresh 0.005% citric acid in 200 ml of 0.019% formaldehyde at 25 °C. On development gels were washed repeatedly in distilled water.

Immunoblot analysis

This was based on the method of Towbin et al. (1979) as described by Hancock and Poxton (1988) with Bio-Rad buffers and substrate. Electrophoretic transfer of LPS to nitrocellulose sheets of a 0.2 μm pore size (Schleicher and Schuell) was

performed at 12 V in electroblotting apparatus for 16 h at 4°C. After transfer, the nitrocellulose membrane was washed in Tris-buffered saline (TBS) for 10 min and blocked with 3% gelatin to prevent non-specific binding of antibodies. A 1 in 10 dilution of culture supernatant fluids (shown previously to give optimum staining) was added for 3 h, followed by two 10 min washes in Tween Tris-buffered saline (TTBS). After a 60 min treatment with an anti-mouse IgG/M/A -horseradish peroxidase conjugate (Zymed), diluted 1 in 500, membranes were washed in TTBS as before. Freshly prepared HRP colour development reagent was added and left for 30 min before washing in distilled water.

Flow cytometry

Washed, overnight cultures of *E. coli* were resuspended to an A_{525} of between 0.5 and 0.6, equivalent to approximately 1×10^8 cfu/ml. Suspensions (1 ml) were centrifuged at $10{,}000 \times g$ in a Microfuge (Beckman) for 2 min and the pellets resuspended in 1 ml of mAb culture supernate, diluted 1 in 10 in dilution buffer (see ELISA methodology), and incubated for 60 min at 37°C. Samples were washed twice in PBS followed by addition of 0.5 ml, sheep FITC-conjugated anti-mouse IgG (ICN) diluted 1 in 100 in dilution buffer. After a further incubation of 60 min at 37°C, samples were washed twice in PBS and resuspended in 1 ml PBS containing 0.5% formaldehyde. Prepared samples, diluted 1 in 50 in PBS, were analysed in an EPICS 'C' (Coulter Electronics) flow cytometer equipped with a 5 W argon ion laser operating at 500 MW output and exciting at 488 nm. Cells were passed through the beam at approximately 500/s from a standard 76 μm flow cell tip. With the use of gates on the log forward angle light scatter (LFLS) signal, both background noise and clumps of cells were excluded from the analysis. Cells stained with FITC conjugate but no primary antibody provided a background staining level which was set at $1\% \pm 0.5\%$ by adjusting the voltage applied to the green fluorescence log (GFL) photomultiplier tube. A total of 50,000 cells were analysed from each sample and the percentage of cells exhibiting positive staining on GFL was calculated by the EPICS 'Stat Pack' programme.

ELISA

Whole cell ELISA was based on the method of Scott (1988). ELISA strips (Immuno module Polysorp F8, Nunc) were coated with washed bacteria (100 μl/well) from a 16 h culture diluted with coating buffer (0.05 M carbonate/bicarbonate pH 9.6 containing 0.02% sodium azide) to a concentration of 2×10^7 cells/ml. Coating was promoted by centrifugation at $630 \times g$ for 4 min and leaving overnight at room temperature. Plates were washed four times with PBS pH 7.2, containing Tween 20 (0.05% v/v) and sodium azide (0.02% w/v) and were then post-coated with bovine serum albumin (5% w/v BSA) and sodium azide (0.02% w/v) in PBS at 100 μl/well followed by overnight incubation at room temperature. After washing

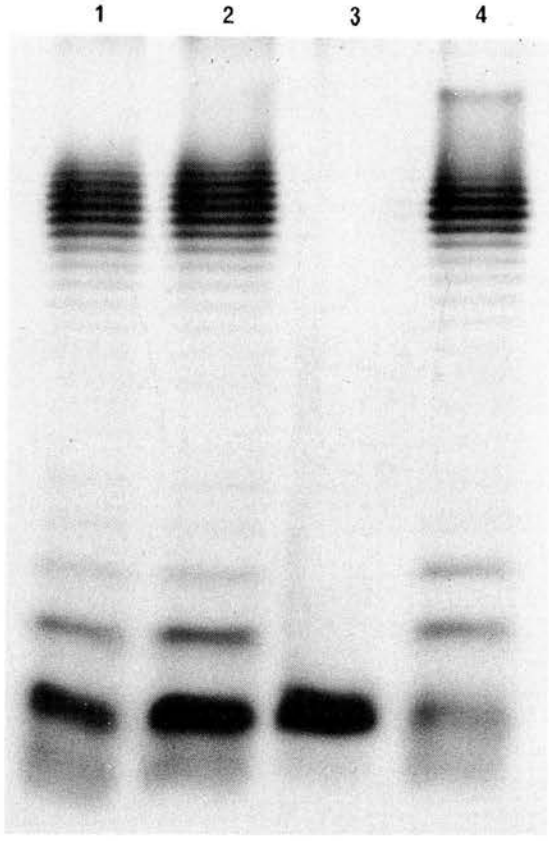

Fig. 1. Silver-stained PAGE of proteinase K digests of *E. coli* grown in filter-sterilised nutrient broth at 37°C for 16 h in an orbital incubator. Track *1*, 018:K1; *2*, 018:K$^-$; *3*, 018rf; and *4*, 06:K5.

four times, plates were rinsed in distilled water and stored at −20 °C until used.

Dilutions of mAb supernatant fluids and conjugate were made in dilution buffer consisting of PBS, with Tween 20 (0.05% v/v), BSA (0.5% w/v), polyethylene-glycol 6000 (4% w/v) and sodium azide (0.02%). Antibody dilutions were added to coated plates at 100 µl/well in triplicate and plates incubated at 37 °C for 90 min before washing four times. Urease-conjugated sheep anti-mouse Ig (Seralab) was diluted 1 in 500, added at 100 µl/well and plates incubated for a further 90 min at 37 °C. Plates were washed four times and rinsed with distilled water before addition of urease substrate (Seralab) at 100 µl/well. Plates were incubated at room temperature and reactions stopped by addition of thimerosal (1%) in distilled water (20 µl/well). Absorbances (A) were read at 590 nm in a Titertek Multiscan.

Results

Silver stain

The silver-stained SDS-PAGE profiles of LPS from the four *E. coli* strains are shown in Fig. 1. The LPS from the three smooth strains showed the characteristic ladder pattern, with each band differing in M_r by one repeating unit of O-antigen. The rough LPS lacked any of the high molecular mass bands which corresponded to LPS substituted with O-polysaccharide chains.

Immunoblots on LPS

Fig. 2. shows the reaction of the anti-LPS antibodies with the four LPS preparations in immunoblots. The 018, O-antigen-specific antibody (mAb 0-1), bound strongly to the high M_r bands of both the smooth 018 strains but not to the 06 LPS (Fig. 2*a*). It also revealed a small amount of high molecular mass O-antigen leaking from the rough mutant of 018. Of the two anti-core mAbs, mAb C-1 bound only to the fast migrating species that corresponded to the core glycolipid demonstrated on silver-stained gels (Fig. 2*b*). Staining was strongest for the rough *E. coli* LPS and weakest for the *E. coli* 06. The reactivity of mAb C-2 again was to the fast migrating core region, but there was also binding to the high M_r, O-antigen bearing molecular species of the smooth strains (Fig. 2*c*).

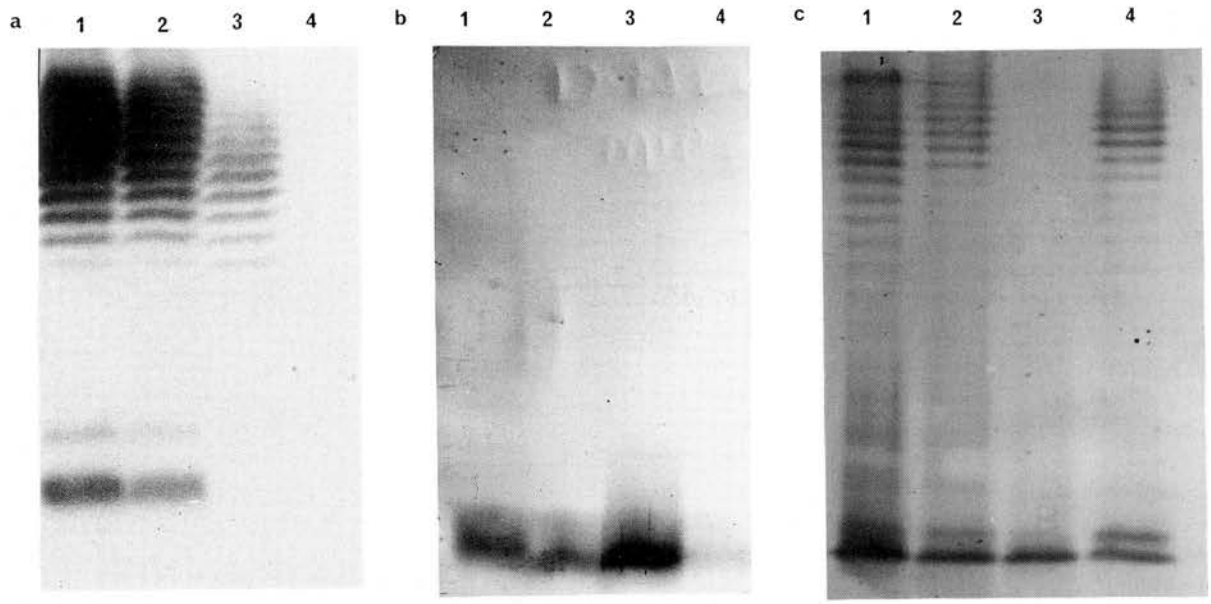

Fig. 2. Immunoblots of proteinase K extracts of *E. coli* strains arranged as in Fig. 1, probed with an anti-018-specific mAb, 0-1 (*a*), and two cross-reactive, core-specific mAbs, C-1 (*b*) and C-2 (*c*).

Flow cytometry on whole bacteria

Fig. 3. shows the binding of anti-LPS mAbs to whole cells by flow cytometry. The reactivity of the 018-specific mAb, mAb 0-1 is shown in Fig. 3*a*. The percentage of bacteria exhibiting positive fluorescence above background levels was in excess of 70% for the two smooth 018 strains, whilst the *E. coli* 06 cells showed negligible binding. The non-capsulate 018 strain showed two distinct populations of cells, indicative of differences in amount of antibody bound. The presence of small amounts of O-antigen on the rough mutant is again evident. The three smooth strains bound anti-core mAb C-1 and mAb C-2 antibodies at less than 10% above background levels (Figs. 3b and 3c). However, binding of mAb C-1 to 018:K$^-$ was almost double that of 018:K1. Percentage binding to the rough mutant by mAb C-1 was 84% compared to a much lower figure of 14% for mAb C-2.

ELISA on whole bacteria

The binding activities of the three mAbs to whole cells in a urease ELISA system are shown in Fig. 4. Reactivity of the 018-specific mAb 0-1 mirrored results of immunoblotting and flow cy-

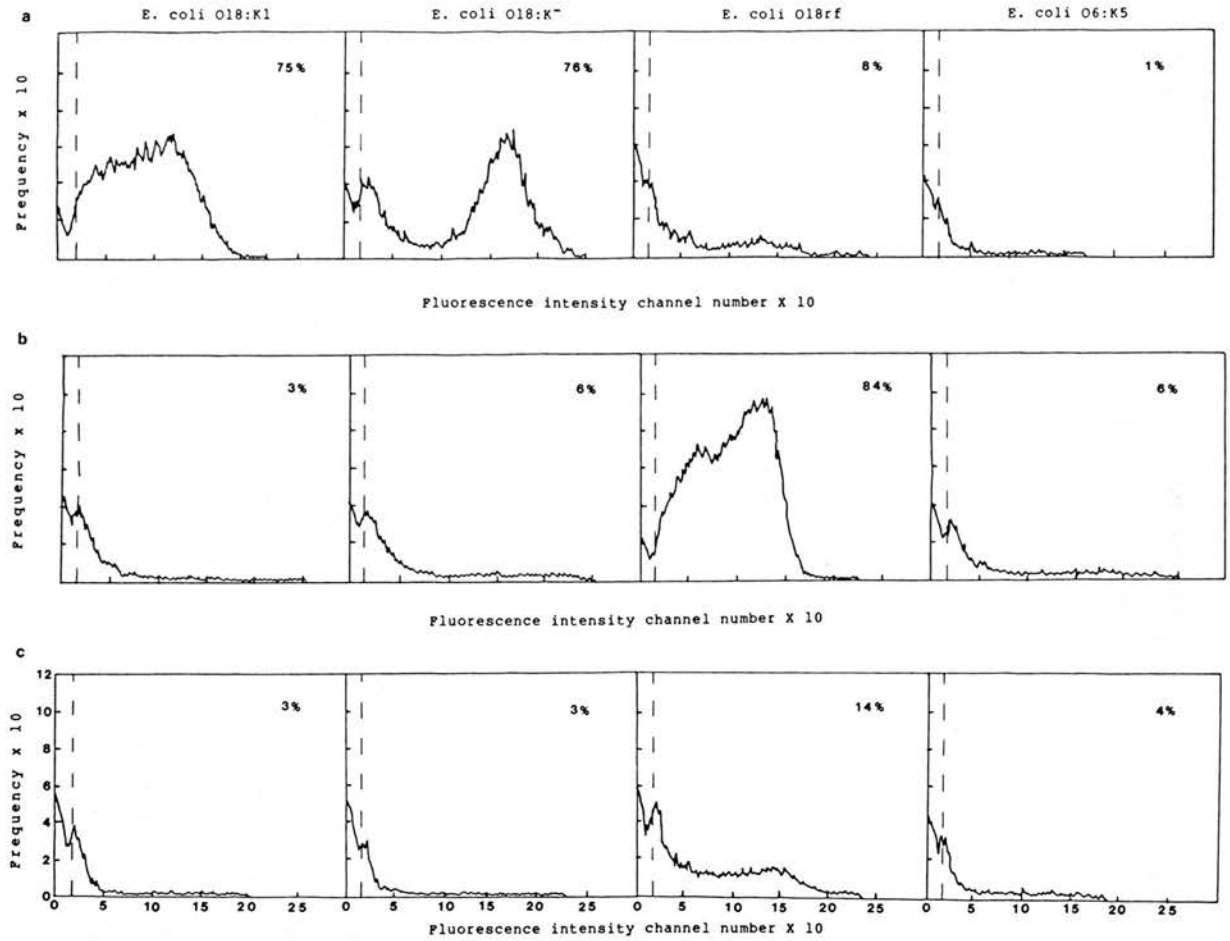

Fig. 3. Flow cytometry. Green fluorescence intensity histograms of culture supernates of three mAbs against whole cells of four *E. coli* strains, (018:K1; 018:K$^-$; 018rf and 06:K5). *a*, *b* and *c* represent binding of the anti-018-specific mAb 0-1, and two cross-reactive, core-specific mAbs, C-1 and C-2 respectively. Percentage values represent bacteria exhibiting positive fluorescence above background levels (indicated by vertical dotted line).

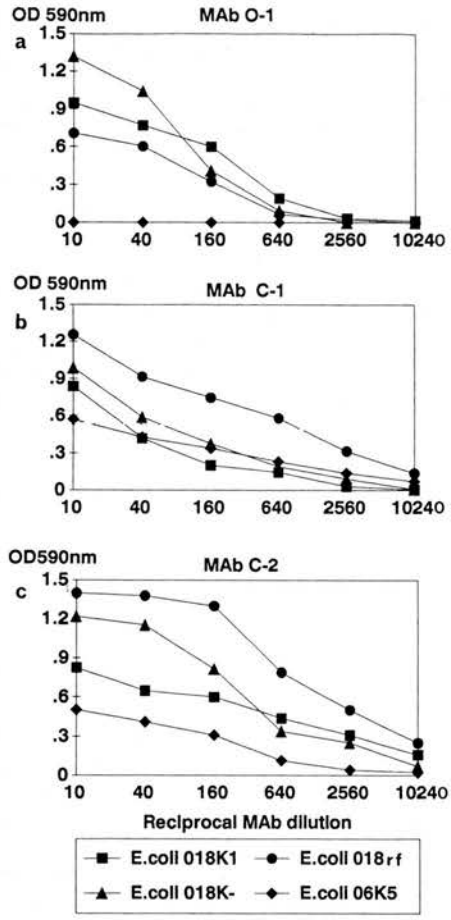

Fig. 4. Binding activities of culture supernates of three mAbs against whole cells of four *E. coli* strains in ELISA: 018:K1; 018: K^-; 018rf and 06:K5. *a*, *b* and *c* represent binding of the anti-018-specific 0-1, and two cross-reactive, core-specific mAbs C-1 and C-2, respectively.

tometry. Smooth 018 strains had the highest A_{590} readings, whilst binding to the rough mutant reflected the presence of some leaking O-antigen. The binding of the mAb at high concentrations was much stronger to the non-capsulate strain than to the capsulate parent. The three smooth *E. coli* strains all showed lower A_{590} values compared to the rough *E. coli* when probed with dilutions of the two anti-core mAbs, C-1 and C-2. Again, the 018 non-capsulate strain showed stronger affinity for the anti-core mAbs compared to its capsulate parent.

Discussion

The individual techniques used in this study provide a means of assessing the binding specificity of anti-LPS mAbs. In combination, a much more comprehensive picture can be built up of the accessibility of endotoxin sites to antibody. Immunoblot patterns of proteinase K LPS extracts against mAbs provide general information regarding their specificity. Indeed, the different reactivity patterns of the two anti-core antibodies was only apparent by immunoblotting. The ladder pattern or single core band effect reflected the binding of C-2 to substituted and unsubstituted core, and C-1 to unsubstituted core material only. It should be noted that the binding of mAbs to blotted LPS does not suffer from the loss or denaturation of epitopes in the same way as proteins.

Results obtained by flow cytometry and ELISA on whole bacteria showed that the absence of O-polysaccharide side chains on the rough mutant appeared to increase the accessibility of core LPS to antibodies, resulting in better binding than to smooth strains. This shielding effect by O-antigenic chains of LPS was also demonstrated by Gigliotti and Shenep (1985). A comparison of the ability of 018:K1 and 018:K^- to bind antibody suggests that capsule may have a role in masking LPS binding sites. Flow cytometry, however, also reveals further information about antibody binding to LPS not detected in ELISA. Within a population of cells showing positive fluorescence an indication of the affinity of an antibody for its antigen can be obtained. The presence of two distinct populations of *E. coli* 018:K^- cells when probed with 0-1 indicates possible differences in structure or amount of LPS.

Flow cytometry offers a means of investigating surface properties of individual cells in large numbers with great speed and efficiency. The technique also has the added advantage of analysing cells in their natural form, devoid of potential distorting influences present in other methods. However, despite the unequivocal potential of flow cytometric analysis of bacteria, it remains an under-used tool, still in its infancy. Phillips and Martin (1988) used flow cytometry for the specific detection of bacteria in aqueous samples. Their

work established problems with background noise as a result of stray light scatter in the optical system, or possibly signals from sub-micron particles in sheath fluids and PBS which had not been removed by filtration. Selective gating near the origin of the cytogram was not feasible in Philips and Martin's study, although it was used in our study to eliminate background noise.

ELISA is an established method for the detection of antibodies to bacteria, offering advantages of speed, flexibility and quantitative accuracy. The apparent higher binding of anti-LPS mAbs to smooth cells in ELISA than to flow cytometry may reflect the better sensitivity of the whole cell ELISA. However, in contrast to flow cytometry, recent data (not shown) suggested that in some instances the expression of coated whole cells on ELISA plates differs from whole cells in suspension, probably due to their alteration during binding manipulations. Our results suggest that ELISA has tended to over-emphasise the binding of anti-core mAbs to whole cells. Indeed, Aydintug et al. (1989) established that both the physical state of bacteria and type of assay used affect the cross-reactivity of an antibody.

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Monoclonal Antibodies as Probes for Detecting Lipopolysaccharide Expression on *Escherichia coli* from Different Growth Conditions

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Running title: Detection of LPS by monoclonal antibodies

Index entries:

Lipopolysaccharide detection by Monoclonal Antibodies.

Lipopolysaccharide expression in Escherichia coli from different growth conditions.

Escherichia coli lipopolysaccharide, detection and expression.

Monoclonal antibodies for detecting lipopolysaccharide in Escherichia coli.

Summary

Monoclonal antibody (MAb) probes were used to investigate the expression of lipopolysaccharide (LPS) on four *Escherichia coli* strains, grown under a variety of conditions in batch culture which mimicked some of the *in vivo* environmental conditions of an infected host. Techniques of silver-staining, immunoblotting, whole cell ELISA and flow cytometry were all used to monitor the expression of LPS on the bacteria and the binding of the anti-LPS MAbs. Growth in heat-inactivated sheep serum and magnesium depleted conditions demonstrated increased expression of LPS core and subsequent increased binding of anti-core MAbs. Magnesium-depleted conditions also resulted in decreased production of O-polysaccharide material. Iron-depleted bacteria showed only minor changes in LPS expression, although increased binding of anti-core MAbs was observed. Nitrogen deficient/high carbon conditions, chosen to promote capsule production, resulted in increased expression of O-polysaccharide and decreased binding of anti-core MAbs. The significance and possible implications of these observations in relation to the use of anti-LPS MAbs as immunotherapeutic agents for endotoxic shock are discussed.

Introduction

The potential of antibody therapy for the prevention and treatment of Gram-negative sepsis and endotoxaemia with anti-lipopolysaccharide (LPS) antibodies has been intensively investigated recently. Much attention has been focussed on the production and characterisation of monoclonal antibodies (MAbs) to common epitopes of endotoxin in the LPS core. There are many reports in the literature of the development of such MAbs (Ziegler et al., 1991; Jongh-Leuvenink, 1990; Mayoral & Dunn, 1990; Pollack et al., 1989). The therapeutic potential of anti-LPS MAbs is however open to much debate (Heumann et al., 1991; Aydintug et al., 1989; Chia et al. 1989). Concern exists as to whether it is possible for these antibodies to bind to sites deep within the LPS molecule, either when the LPS is bound to the bacterium or to host components such as high density lipoprotein and endothelial surfaces, or "free" in micelles. A further complication exists by which the heterogeneity of O-polysaccharide chain length and degree of substitution of the LPS core may affect antibody accessibility (Gigliotti & Shenep, 1985).

We have shown recently that it is possible, with a combination of ELISA and flow cytometry, to use monoclonal antibodies to detect LPS expression on whole bacteria (Nelson et al., 1990). Results showed however, that although anti-O-polysaccharide MAbs could readily be observed binding to bacteria by both techniques, anti-core MAbs could be shown to bind significantly only by the sensitive ELISA and not by flow cytometry.

It is now well recognised that the growth environment of bacteria greatly influences the phenotypic expression of surface characters (Smith et

al., 1991; McGroarty & Rivera, 1990; Kelly et al., 1989; Morse et al., 1983). This study attempts to investigate with MAb probes the expression of LPS on bacteria cultured in conditions which mimic those *in vivo*.

Methods

Bacteria. Four strains of *Escherichia coli* were kindly supplied by Dr.A.S. Cross, Walter Reed Institute for Army Research, Washington, DC, U.S.A. They consisted of two clinical isolates (O18:K1 and O6:K5 serotypes) together with two isogenic mutants from the O18 parent: a non-capsulate mutant ($O18:K^-$) and a rough mutant (O18Krf).

Culture conditions. The following growth media were used: a) Nutrient broth (Gibco): filter-sterilised. b) Magnesium depleted: prepared as a modification of the Malka minimal medium of Robert-Gero et al. (1979) as follows: solution A, $NaHPO_4$ (73.4 mg ml^{-1}), KH_2PO_4 (32.4 mg ml^{-1}); solution B, $MgSO_4.7H_2O$ (20.5 mg ml^{-1}); solution C, 20% w/v glucose; solution D, $FeSO_4.7H_2O$ (1.83 mg ml^{-1}) in sterile distilled water to which one drop of concentrated hydrochloric acid was added; solution E $(NH_2)_2SO_4$ (50.0 mg ml^{-1}). All chemicals were from BDH. Solutions were prepared with sterile distilled water and were filter sterilised. All solutions except C were stored over chloroform. To prepare one litre of Malka, 20ml A, 20ml B, 20ml C, 1ml D and 20ml E added to 919ml sterile distilled water. A magnesium depleted medium contained 0.17 mmol. L^{-1}, prepared by the addition of only 1% volume (0.2ml) of solution B used for the standard minimal medium. c) Nitrogen deficient/high carbon medium: prepared following the method of Sutherland & Wilkinson (1965)

containing: 1g yeast extract (Oxoid); 1g casamino acids (Difco technical grade); 10g Na_2HPO_4; 3g KH_2PO_4; 0.2g $MgSO_4.7H_2O$; 11g K_2SO_4; 1g NaCl; 0.01g $CaCl_2$; and 0.01g $FeSO_4$. The volume was made up to 1000ml with sterile distilled water and autoclaved. To this, 20% w/v filter-sterilised glucose solution was added to give a final concentration of 2% glucose w/v. d) Iron depleted: prepared by the addition of 150 μmol. L^{-1} 2,2' dipyridyl to Gibco nutrient broth. e) Heat inactivated sheep serum (obtained from the Moredun Research Institute, Gilmerton Road Edinburgh): serum was filter-sterilised (0.45μm pore size) and heat inactivated at 56°C for 60 min and stored at -20°C.

Log phase starter cultures were prepared by growing in nutrient broth at 37°C for 4h before harvesting and washing twice in phosphate-buffered saline, pH 7.4 (PBS). A 1% inoculum was added to each growth medium. Cultures were grown at 37°C in 100 ml of each medium to early stationary phase in an orbital incubator prior to harvesting and washing as above.

Monoclonal antibodies. MAbs were prepared by fusing spleen cells from immune BALB/c mice with NS-O myeloma cells by standard techniques (Kipps and Hertzenberg, 1986). The reactivity of the MAbs was determined by the LPS-polymyxin ELISA method of Scott and Barclay (1987) as described by Nelson et al (1990). Full details of the immunisation of mice, selection of hybridomas and characterisation of MAbs are to be published elsewhere. Three MAbs with known specificities in LPS-polymyxin ELISA were selected for this study: MAb O-1, specific for O18 O-antigen, and two cross-reactive anti-core MAbs (C-1 and C-2), reactive to either core not substituted with O-antigen (C-1) or to both substituted and unsubstituted core material (C-2) (for more details see Nelson et al., 1990). Supernatant fluids of hybridoma cell cultures grown in RPMI 1640 supple-

mented with 5% (v/v) fetal calf serum in 150 cm^2 flasks were used throughout. Cell cultures were grown to maximum cell density and harvested at 50% cell viability. These three hybridomas gave yields of approximately 50 μg/ml.

Preparation of LPS. LPS was prepared from whole washed bacteria by the proteinase K method of Hitchcock and Brown (1983) as described by Hancock and Poxton (1988). For each growth condition the density of washed bacteria was adjusted to A_{525} of 0.5 prior to proteinase K treatment. This allowed direct comparison of cells from each growth medium.

PAGE. PAGE was performed on 14% slab gels with the Laemmli buffer system (Laemmli, 1970), except SDS was omitted from the stacking and separating buffers. Samples (10 μl for silver stain, or 20 μl for immunoblotting) of the proteinase K LPS extracts were loaded onto the gels. The LPS separating gels were stained with silver by a method developed by Tsai and Frasch (1982), modified by Hancock and Poxton (1988).

Immunoblot transfer. LPS separated by PAGE was electroblotted to nitrocellulose by the method of Towbin et al (1979) as described by Hancock and Poxton (1988) with BioRad buffers and substrate. Nitrocellulose membrane of 0.2 μm pore size was obtained from Schleicher and Schuell and an anti-mouse IgG-Horseradish peroxidase conjugate used (ICN). Antibody culture supernates were diluted 1 in 10.

Diluents and buffers used in ELISA. (1) Coating buffer consisted of 0.05M carbonate/bicarbonate, pH 9.6. (2) Post-coat buffer consisted of

phosphate-buffered saline pH 7.2 (PBS) containing 5% (v/w) bovine serum albumin (BSA) (ICN Biomedicals). (3) Wash buffer consisted of PBS containing 0.05% (v/v) Tween 20. (4) Dilution buffer consisted of PBS containing 0.05% (v/v) Tween 20, 0.5% BSA and 4% (w/v) polyethylene glycol 6000 (Sigma Chemicals).

All solutions contained 0.02% sodium azide.

ELISA procedure. ELISA strips (Immuno module Polysorp F8, Nunc) were coated with washed bacteria after measuring optical density (OD) at 540nm and diluting with coating buffer to a concentration of $2x10^7$ cells ml^{-1}. Coating was promoted by centrifugation at 630g for 4 min and leaving overnight at room temperature. Plates were washed four times in wash buffer, before post-coating with post-coat buffer at 100μl per well overnight at room temperature. After further washing, plates were rinsed in distilled water and stored at -20°C until used.

MAb supernatant fluids were diluted in dilution buffer and added to coated microplates at 100μl per well in triplicate. Plates were incubated at 37°C for 90 min before washing four times with wash buffer. Urease-conjugated sheep anti-mouse IgG (Seralab) was diluted 1 in 500, added at 100μl per well and plates incubated for a further 90 min at 37°C. Plates were washed four times and rinsed in distilled water before urease substrate (Sera Lab) at 100μl per well was added. Plates were incubated for 60 min at room temperature and reactions stopped by adding 1% w/v thimerosal (Sigma) in distilled water (20μl per well). The OD of wells was read at 590nm on an automated microplate reader Titertek Multiscan (MC, Flow Laboratories). Final results were expressed after subtraction of the OD of negative control wells (coated with BSA post-

coat only) for each MAb.

Flow cytometry. Flow cytometry was based on the method described by Nelson et al (1990). Briefly, washed cultures of bacteria were resuspended to a concentration of ca. 1x 10^8 cells ml^{-1}. Pellets from 1 ml were resuspended in 1 ml of MAb culture supernate, diluted 1 in 10 in dilution buffer (see ELISA methodology), and incubated for 60 min at 37°C. After washing twice in PBS, 0.5 ml sheep FITC-conjugated anti-mouse IgG (ICN), diluted 1 in 100 in dilution buffer, was added and incubated for 60 min at 37°C. After further washing in PBS, the pellet was resuspended in PBS containing 0.5% formaldehyde. Samples were diluted 1 in 50 in PBS and analysed in an EPICS "C" (Coulter Electronics) flow cytometer with a 5 watt argon ion laser operating at 500 mW and exciting at 488 nm. 50,000 cells at 500 cells.min^{-1} were passed through the beam from a 76 μm tip. Background noise and clumps of cells were excluded by a gate on the log forward angle light scatter. Percent cells exhibiting positive staining were calculated with the EPICS "Stat Pack" programme.

Results

Silver-staining and Immunoblot Analysis of Lipopolysaccharide.

Figs. 1-3 represent silver-stained PAGE profiles of LPS and their corresponding immunoblots from four *E. coli* strains grown under different batch culture growth conditions. The three smooth strains showed the characteristic ladder pattern, each step up representing LPS substituted with a progressively increasing number of O-polysaccharide repeating oligosaccharide units. These were missing from the rough mutant. The effect of each growth condition on the expression of LPS was compared

with nutrient broth. Silver-stained profiles of LPS of proteinase K digested whole cells grown in nutrient broth, iron-depleted and nitrogen deficient/glucose enriched media are shown in Fig. 1a. LPS from iron-depleted cells show only minor changes compared to the LPS from nutrient broth grown cells, whilst cells grown in a nitrogen deficient/glucose enriched medium showed an increased expression of both mid-range and high molecular mass bands. Immunoblotting of transblotted gels against MAb O-1 (Fig.1b), demonstrates the specificity for the O-antigen of *E. coli* O18 and small amounts of high molecular mass O-antigen leaking from the rough mutant of O18. The immunoblot again illustrates greater expression of O-antigen as well as an overall increase in O-polysaccharide chain length for cells grown under nitrogen deficiency. Probing with C-2, an anti-core MAb reactive against both substituted and unsubstituted core material showed no significant differences between growth conditions (Fig.1c). PAGE of LPS from the four magnesium-depleted *E. coli* strains show a significant increase in the expression of unsubstituted core-glycolipid material and other low molecular mass bands (Fig.2a). Immunoblotting with MAb O-1 showed similar expression of O-antigen for both nutrient broth and magnesium-depleted conditions (fig 2b), whilst probing with MAb C-2 further highlights a pronounced increase in core LPS (Fig.2c). Growth in sheep serum resulted in greater expression of both the fast migrating core region, as well as high M_r, O-antigen bearing molecular species of the smooth strains compared to growth in nutrient broth (Fig.3a). Immunoblot analysis with MAb C-1, reactive only with unsubstituted core material, and C-2 , demonstrates better expression of predominantly rough form LPS when grown in serum (Figs.3b,c).

ELISA on whole bacteria.

The binding activities of the three MAbs to whole cells of *E. coli* strains O18:K1 and O18:K$^-$, cultured under various growth conditions are shown in (Fig.4). No significant differences in binding of the anti-O MAb to *E. coli* O18:K1 were detected for the different growth conditions (Fig.4a). However, the non-capsulate mutant *E. coli* O18:K$^-$ showed a significant decrease in binding of MAb O-1 to cells grown under both iron and magnesium limitation (Fig.4b). The O18 non-capsulate strain showed stronger affinity for both anti-core MAbs compared to its capsulate parent when grown under each growth condition Figs. 4c-f. Growth of both *E. coli* strains in the nitrogen deficient/glucose enriched medium resulted in lower binding of the two anti-core MAbs compared to growth in nutrient broth. Significant increases in OD_{590} when both strains were grown in serum and magnesium limitation and probed with MAbs C-1 and C-2 were observed. A similar, yet slightly lower increase in binding of anti-core MAbs was seen for growth under iron-limitation compared to nutrient broth.

Flow cytometric analysis.

The effect of growth conditions on the expression of LPS on whole bacteria was also investigated by flow cytometry. Flow cytometric profiles, which relate to the intensity of fluorescence signal (ie antibody binding to bacteria), were obtained consistently in three separate experiments. Examples of representative profiles are illustrated in Fig. 5. The histograms produced by the interaction of the O18-specific MAb, O-1, and cells grown in nutrient broth and a magnesium depleted medium are shown in Fig. 5a. A 41% decrease in magnesium-depleted bacteria exhibiting positive fluorescence above background

levels compared to nutrient broth cells was observed. The biphasic fluorescence pattern produced by magnesium-depleted cells demonstrated the presence of two distinct subpopulations of *E.coli* O18:K1 on the basis of differential binding by MAb O-1. Probing the same cells with C-1, a MAb reactive against unsubstituted core LPS, resulted in positive fluorescence levels of 5% for nutrient broth and 29% for magnesium depletion (Fig. 5b). Significant increases in the binding levels of both anti-core MAbs, C-1 and C-2 (a MAb reactive against both substituted and unsubstituted core LPS) were also observed when *E.coli* O18:K^- cells were grown in serum compared to nutrient broth (figs. 5c,d).

Flow cytometry data, showing the effect of growing all four *E.coli* strains under the different growth conditions on MAb recognition of LPS associated epitopes is presented in Table 1. Although relative differences between growth conditions remained constant, day to day variation in percentage labelling within a given sample was evident. Smooth cells grown under serum and magnesium-depleted conditions again showed the largest increases in binding of anti-core MAbs. Iron-depleted and nitrogen deficient cells showed only minor differences compared to nutrient broth grown cells. Probing cells with the O-antigen specific MAb resulted in similar binding levels for most conditions, whilst magnesium depleted cells, notably O18:K^- showed a significant decrease. Enhanced binding of both anti-core MAbs, especially C-1, was observed against the rough mutant, O18:Krf, compared to the smooth strains. Minor variations between growth conditions of O18:Krf cells reacted with anti-core MAbs favoured higher percentage labelling for bacteria grown in magnesium-depleted conditions.

Discussion

The environmental modulation of cell surface components of both Gram-positive and Gram-negative bacteria has been intensively investigated (Brown & Williams, 1985). Among the environmental factors that commonly influence the properties of microbial cells, the availability of essential nutrients assumes particular importance (Harder & Dijkhuizen, 1983). LPS is a major cell surface component of all Gram negative bacteria, and implicated as a cause of endotoxic shock (Ryan, 1985). This study has examined the effects of a number of nutrient growth conditions relating to those *in vivo*, on the accessibility and expression of LPS antigens using MAbs.

Our results indicated that the expression of *E.coli* core LPS was increased when grown in magnesium depleted conditions (0.17 mmol. L^{-1}, cf. 0.65-1.0 mmol. L^{-1} in serum). This was reflected in greater binding of anti-core MAbs using techniques of immunoblotting, whole cell ELISA and flow cytometry. Magnesium is important in maintaining the stability of the structural arrangement of larger molecules such as LPS within the outer membrane of Gram-negative bacteria (Costerton et al., 1974). Cell walls of magnesium-limited cells of *Bacillus subtilis* showed an increased Mg^{2+} binding affinity over magnesium-sufficient cells (Meers & Tempest, 1968). Thus, certain organisms may respond to magnesium limitation by improving their ability to bind the ions by increasing negatively charged cell surface components such as LPS. Using both batch and continuous culture Day & Marceau-Day, 1982, also reported compositional changes in *Pseudomonas aeruginosa* LPS in response to magnesium ion concentration, reflecting functional alterations in the LPS. Data indicated a change in core size of LPS relative to the O-antigen component, suggesting the possibility of magnesium having a regulatory role on one

or more of the LPS biosynthetic enzymes. An increased production of low molecular mass LPS was seen for *E. coli* grown at intermediate growth rates under magnesium limitation (Dodds et al. 1987). The amino-sugar, 2-aminohexose was also found to be absent from the O-polysaccharide. Flow cytometric analysis of *E.coli* O18:K1 grown under magnesium-depleted conditions and probed with an O-antigen specific MAb revealed two populations of bacteria. Possible reasons for this include: differences in the structure or amount of LPS; the amount of capsule (shown to be inhibited under these conditions by Taylor et al. 1981), or morphological heterogeneity of bacteria grown under magnesium-depletion. The composition of LPS from *E. coli* O18:K$^-$ LPS was shown to differ from nutrient broth grown LPS using nuclear magnetic resonance spectrometry (unpublished data). As well as a decrease in the amount of O-polysaccharide, rhamnose, a constituent of the O18-O-antigen, was found to be replaced by an as yet unidentified sugar.

The low availability of iron is acknowledged as a key determinant of virulence (Griffiths, 1987). Alterations in the outer membrane of many bacterial species under conditions of iron-deprivation include the production of high molecular mass, iron regulated outer membrane proteins (Neilands, 1982). Little effect of iron-depletion on LPS production was observed by silver-staining and immunoblotting. However, increasing the degree of iron-deprivation led to a greater expression of rough core at the expense of high molecular mass O-polysaccharide LPS (data not shown). Indeed, binding of anti-core MAbs to whole cells was shown to improve by whole cell ELISA (Fig. 2), although not significantly using flow cytometry (Table 1). This may be partly

explained by differences in the two techniques. Whilst ELISA may offer greater sensitivity, flow cytometry allows the analysis of cells without exposure to distorting influences present in other methods such as ELISA, leading to improved accessibility to LPS antigens. Although changes in the expression of LPS per-se have been illustrated, growth under stress conditions such as iron and magnesium are likely to alter the structure and composition of other components of the outer membrane. Therefore, LPS core determinants normally showing limited accessibility may become better exposed on the cell surface. Although this study attempted to assess the binding of anti-LPS MAbs to whole cells, release of membrane fragments from cells must also be considered. Indeed, variations in growth conditions could alter the extent and nature of the excretion of such fragments (Hoekstra et al. 1976).

The present findings also show a significant increase in binding of anti-core MAbs when smooth *E. coli*, especially the non-capsulate O18:K$^-$, were grown in heat-inactivated sheep serum (HSS). Immunoblotting, ELISA and flow cytometry all showed marked increases in binding of MAbs to the core region, despite an apparent increase in the expression of O-antigen as revealed by silver-staining. The possible presence of endogenous anti-LPS antibodies within the serum may have caused a false impression of MAb binding. However, since MAbs were raised in a different species to the serum used, antibody conjugates would only recognise mouse MAbs, and removing any anti-LPS antibodies from the serum by absorption had no effect on results (data not shown). Thus HSS appears to alter the antigenic expression of LPS to permit binding of MAbs to the core region. Chedid et al. 1968, proposed an enzymatic process within serum capable of attacking cell wall components, thereby unmasking the conserved rough

antigenic structures.

Although capsule plays an important role in the serum resistance of many bacteria (Leying et al. 1990), it has been reported not to provide a barrier function for binding of anti-O antibodies to *Klebsiella* (Williams et al. 1988) and some *E. coli* including O18 (Cross et al. 1986). Indeed, binding of the anti-O18 MAb to capsulate and non-capsulate strains of *E. coli* O18 showed no effect due to the presence of capsule. However, greater binding of anti-core MAbs to the non-capsulate strain indicates capsules may have a function as a barrier for the penetration of anti-core LPS antibodies. Growth under nitrogen-deficient/high carbon conditions has previously shown to promote capsule formation (Sutherland and Wilkinson, 1965). Reduced binding of anti-core MAbs to both capsulate and non-capsulate bacteria grown under these conditions suggested another factor, other than capsule exerting an influence. Indeed, an observed increase in chain length and production of O-polysaccharide was revealed by silver-staining and immunoblotting (Fig. 1). Since O-polysaccharide is known to reduce accessibility to core LPS (Gigliotti & Shenep, 1985), a change in both its production and possible arrangement may have strengthened this effect. A variety of growth conditions have been shown to alter the expression of O-antigenic LPS molecules. McGroarty & Rivera (1990) demonstrated a dramatic decrease in the length of the O-specific LPS grown under a number of stress conditions, whilst growth phase (Day & Marceau-Day 1982) and growth rate (Dodds et al. 1987) have also influenced its expression. Such variability in the expression of LPS polysaccharide formation may contribute towards a bacterium's ability to adapt to changes in its environment. Since varia-

bles other than nutrient limitation have been shown to influence the expression of LPS, it is acknowledged that in using the batch culture model, the significance of altering one variable can not be divorced with certainty from the influence of others.

The diversity of phenotypic responses to environmental conditions by individual bacteria indicates that the expression of a microbe's genome is selective (Koch, 1976). Microbes generally express only that part of their genome that enables them to become structurally and functionally adjusted to a certain set of conditions (Harder & Dijkhizen, 1983). Implicit in this is the role of DNA supercoiling and its involvement in the environmental regulation of gene expression, reviewed by Dorman (1991).

Variation in the expression of LPS and other outer membrane components in response to a changing environment has important implications for the usefulness of anti-LPS MAbs as immunotherapeutic agents against endotoxic shock. Exposure of increased numbers of specific antigenic epitopes on the bacterial cell surface may increase the potential of MAbs targeted towards conserved regions of the LPS molecule. However, *in vivo*, LPS is thought to be present in a number of different forms (Pollack et al. 1989), and the complex environmental conditions in the infected host will be quite different from most laboratory conditions (Brown & Williams, 1985). Whilst caution is always needed when relating effects of *in vitro* growth to those *in vivo*, the possibility of similar changes in the expression of LPS taking place *in vivo* can not be excluded.

Acknowledgements.

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Legends

Fig.1. (a) Silver stained LPS profiles of proteinase K whole cell digests of 4 *E. coli* strains, (O18:K1, O18:K$^-$, O18:Krf and O6:K5) separated by PAGE (14% w/v acrylamide). (b,c) Immunoblots of the 4 *E. coli* strains transferred to NIC paper and probed with (b) an anti-O18 specific MAb, O-1, and (c) a core-specific MAb, C-2. Tracks 1-4, 5-8 and 9-12 represent 4 *E. coli* strains grown to early stationary phase in nutrient broth, an iron-depleted medium and a nitrogen-deficient medium respectively.

Fig.2. (a) Silver stained LPS profiles of proteinase K whole cell digests of 4 *E. coli* strains, (O18:K1, O18:K$^-$, O18:Krf and O6:K5) separated by PAGE (14% w/v acrylamide). (b,c) Immunoblots of the 4 *E. coli* strains transferred to NIC paper and probed with (b) an O-18 specific MAb, O-1, and (c) a core specific MAb, C-2. Tracks 1-4 and 5-8 represent the four *E. coli* strains grown to early stationary phase in nutrient broth and a magnesium depleted medium respectively.

Fig.3. (a) Silver stained LPS profiles of proteinase K whole cell digests of 4 *E. coli* strains (O18:K1, O18:K$^-$, O18:Krf and O6:K5), separated by PAGE (14% w/v acrylamide). (b,c) Immunoblots of the 4 *E. coli* strains transferred to NIC paper and probed with two core-specific MAbs, C-1 (b) and C-2 (c). Tracks 1-4 and 5-8 represent 4 *E. coli* strains grown to early stationary phase in nutrient broth and heat inactivated sheep serum respectively.

Fig.4. ELISA activity of three anti-LPS MAbs against whole cells of *E. coli* O18:K1 (a,c and e), and O18:K^- (b,d and f), grown under different growth conditions. a and b represent binding of an anti-O18-specific MAb, O-1, and (c and d), and (e and f) represent binding of two core-specific MAbs, C-1 and C-2 respectively. Cells were grown to early stationary phase in nutrient broth, ■., nitrogen deficient medium, ▨., iron depleted medium, □., magnesium depleted medium, ▨, and heat inactivated sheep serum, ▨. Each histogram bar represents the mean optical density value of three separate experiments carried out in triplicate.

Fig.5. Green fluorescence intensity histograms of two anti LPS MAbs against whole cells of O18:K1 grown to early stationary phase in nutrient broth and a magnesium depleted medium (a and b), and O18:K^- grown in nutrient broth and a heat-inactivated sheep serum medium (c and d). a represents the binding of a O18-specific MAb , O-1; and b and c, a core-specific MAb C-1; and d, a second core-specific MAb, C-2. Percentage values represent bacteria exhibiting positive fluorescence above background levels.

Table 1. Flow cytometric analysis of anti-LPS MAb binding to whole cells of four *E. coli* grown under different conditions. Percentage values represent the mean positive fluorescence of bacteria above background levels from three separate experiments. MAbs include an anti-O18, O-polysaccharide (O-1), and two anti-core MAbs (C-1 and C-2).

* = mean ±SD

ND = not done

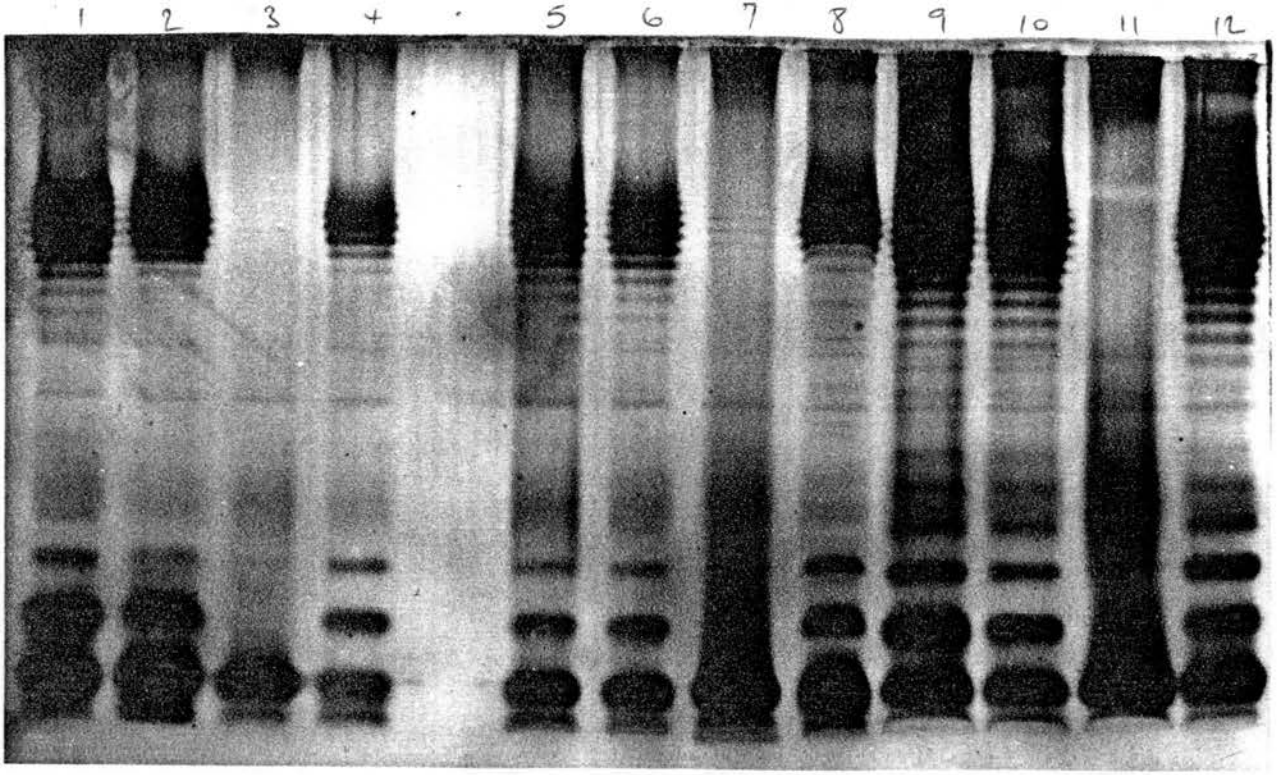

Fig 1a

Fig. 1B

1 2 3 4 5 6 7 8 9 10 11 12

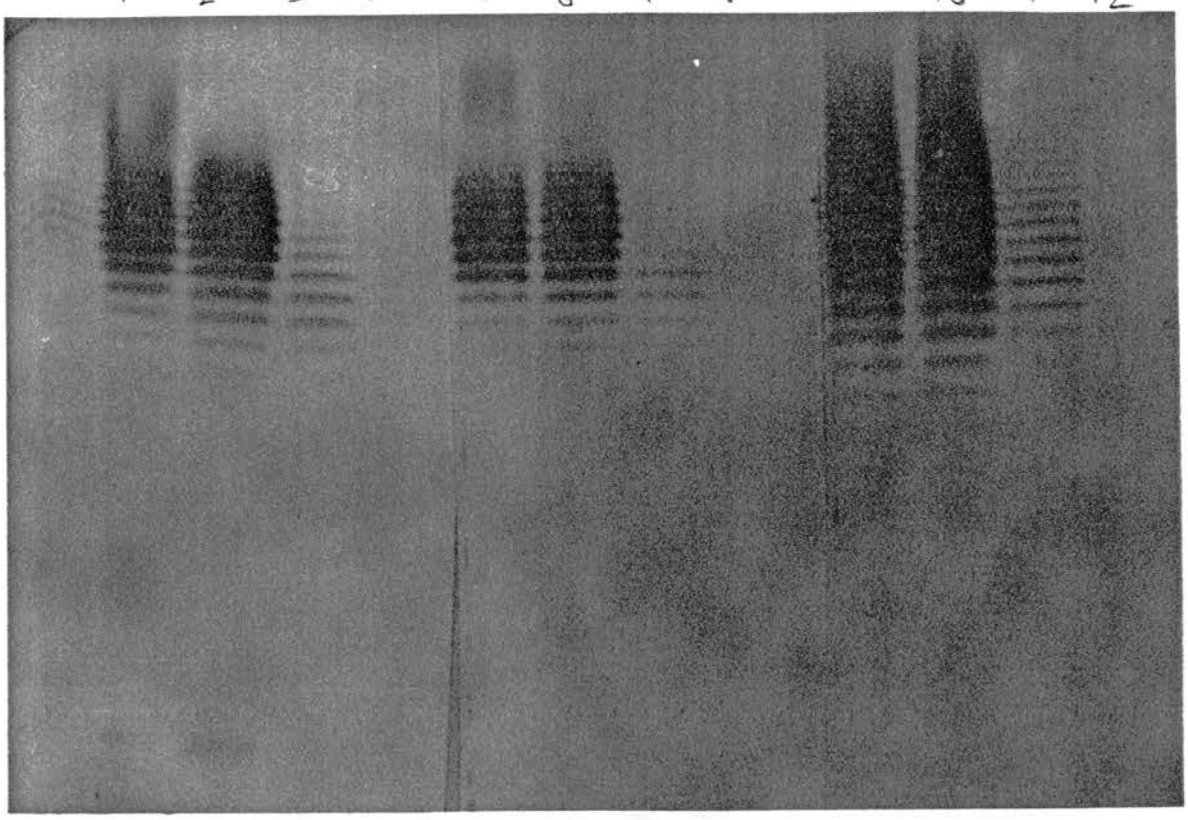

Fig. 1C

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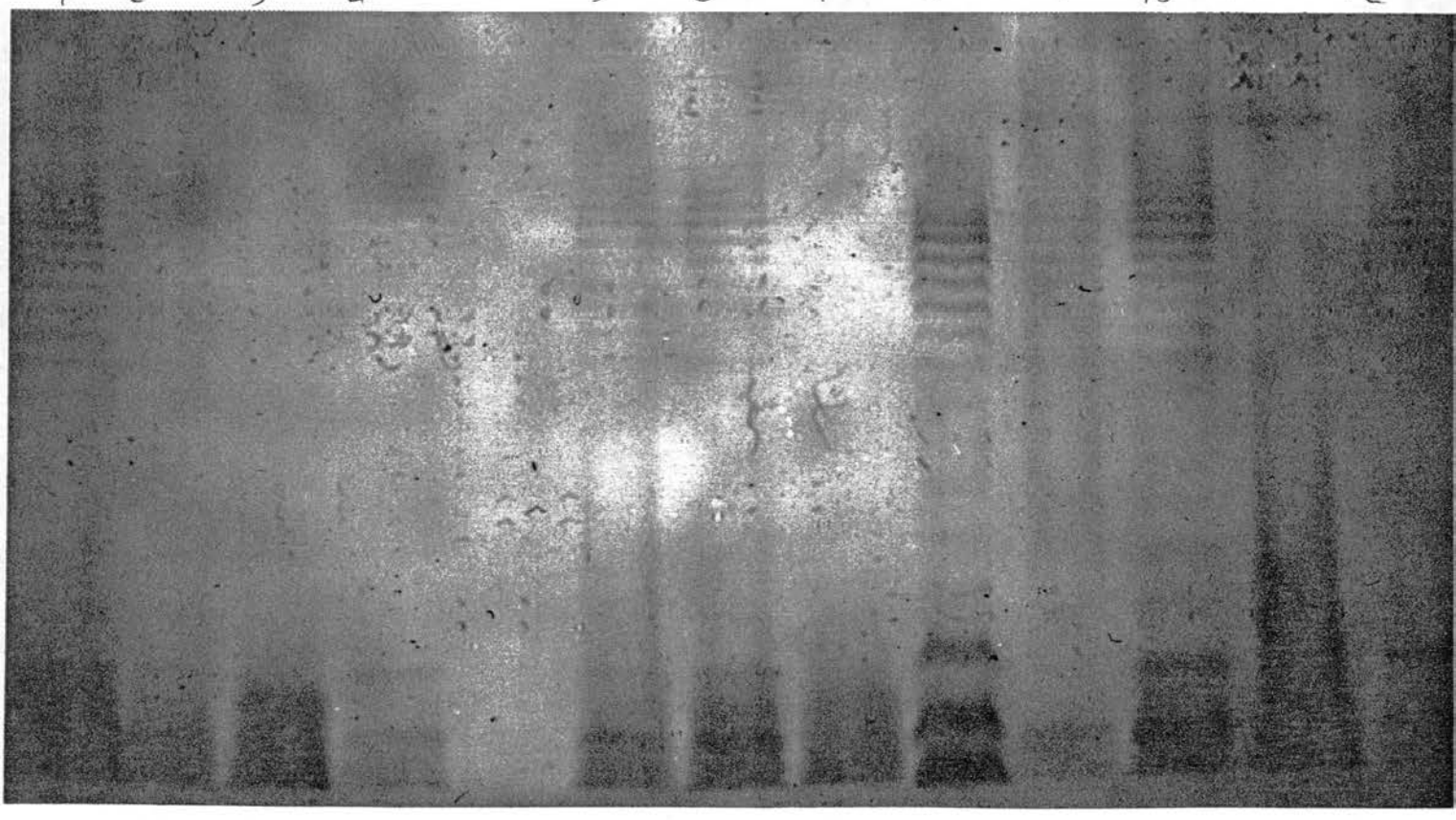


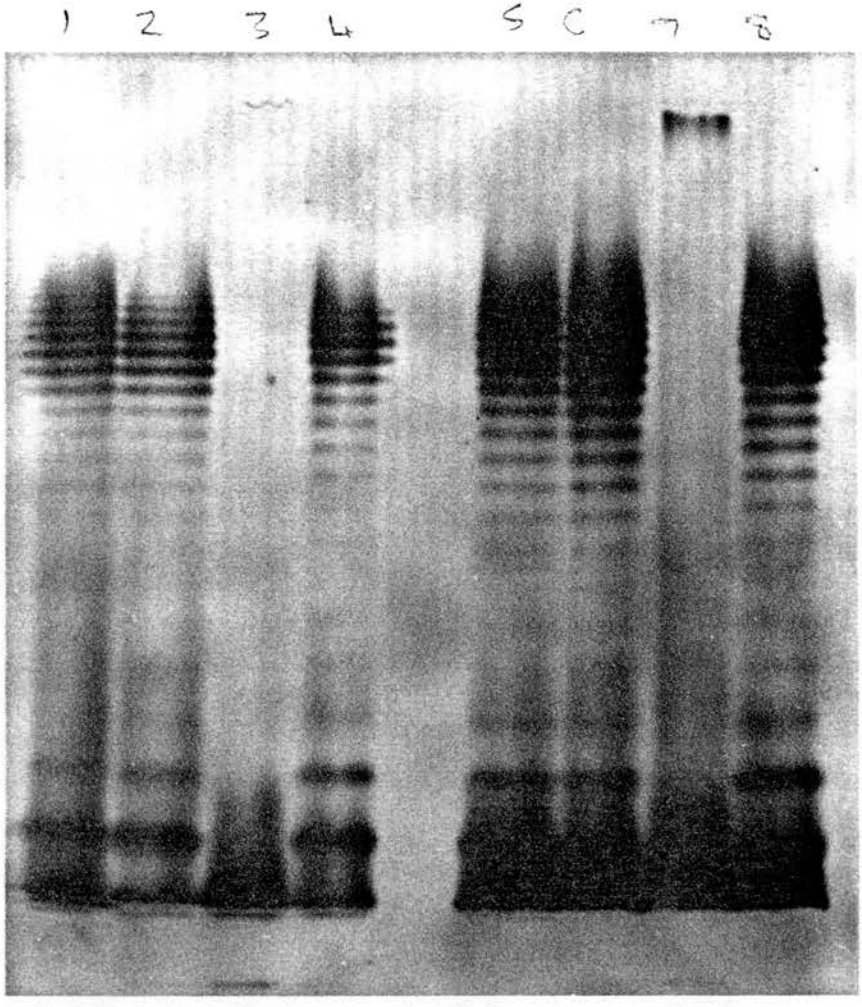


Fig 2a

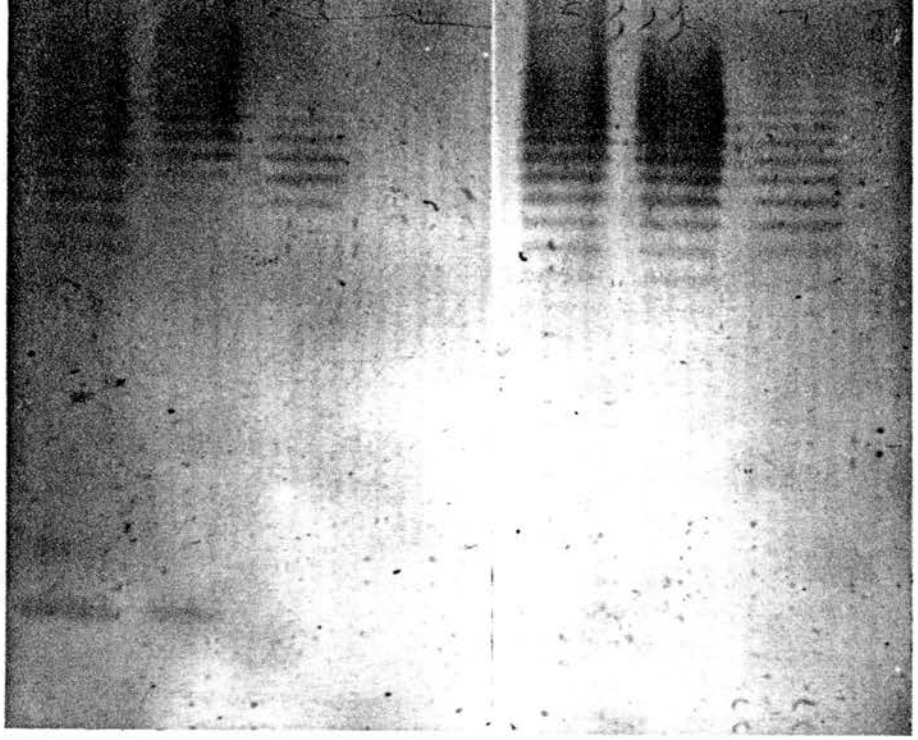
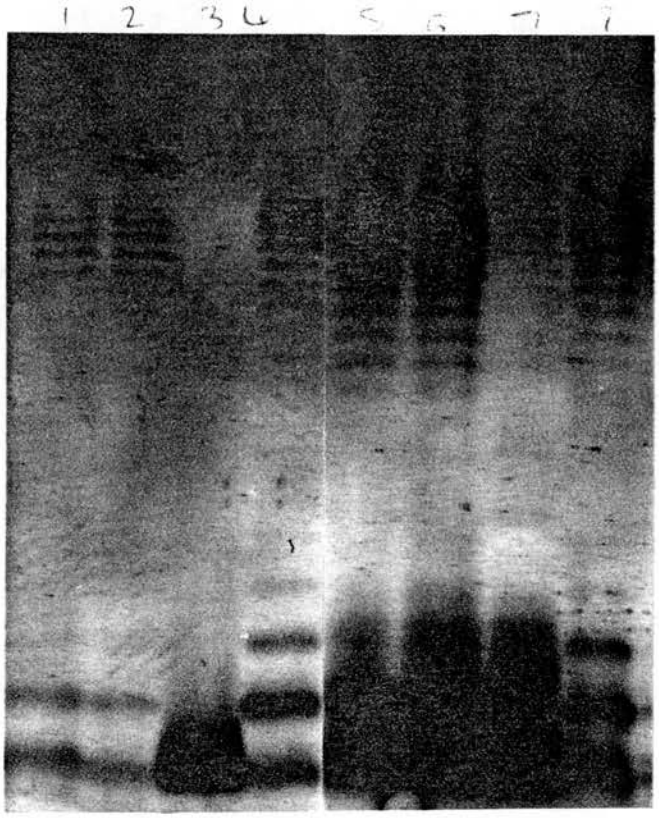


Fig 2b

Fig 2c



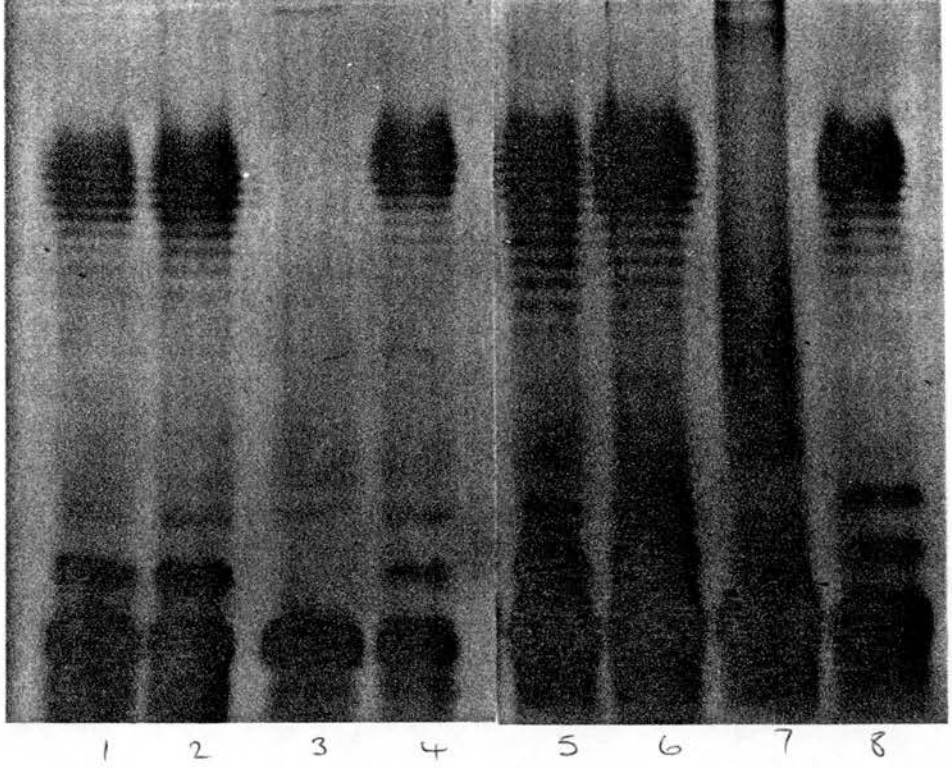


Fig 3a

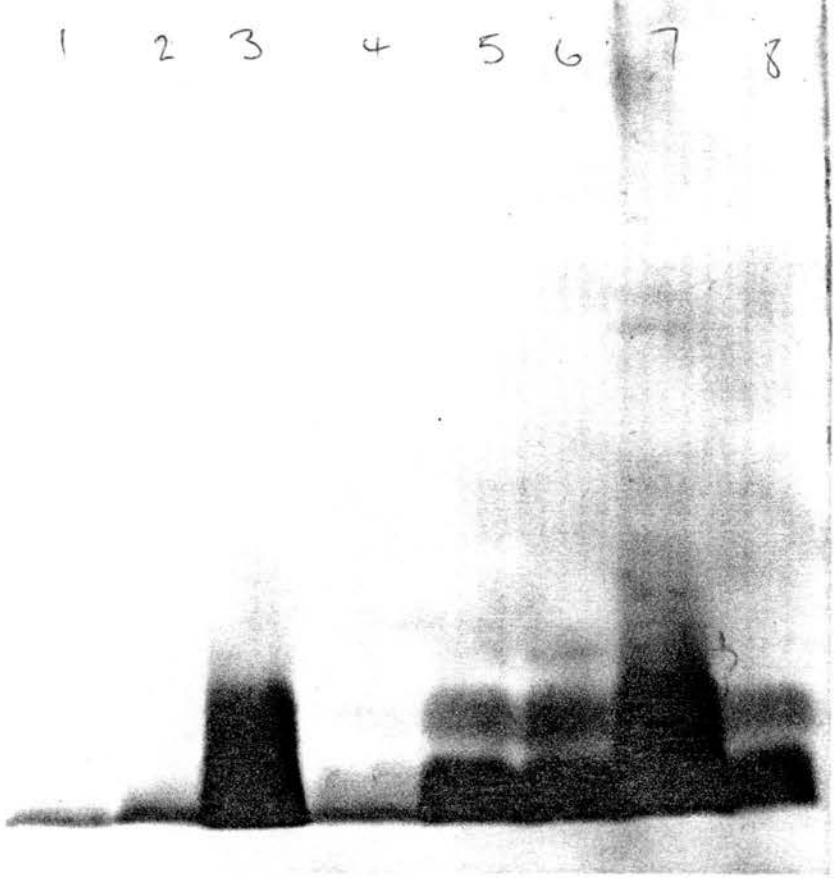
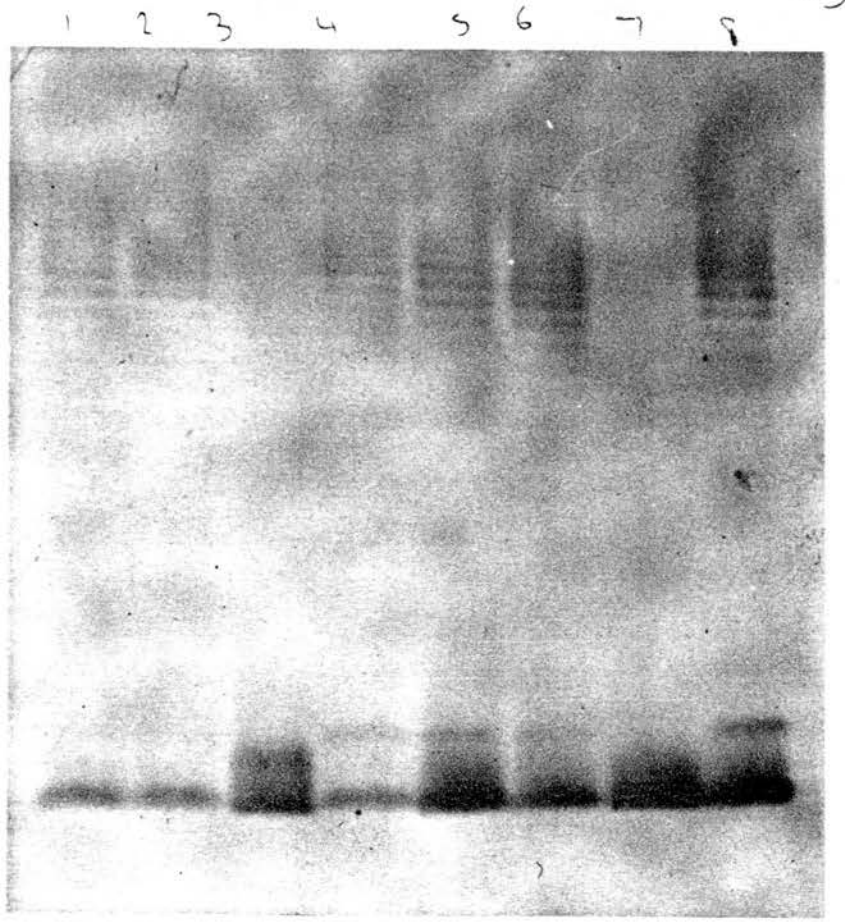
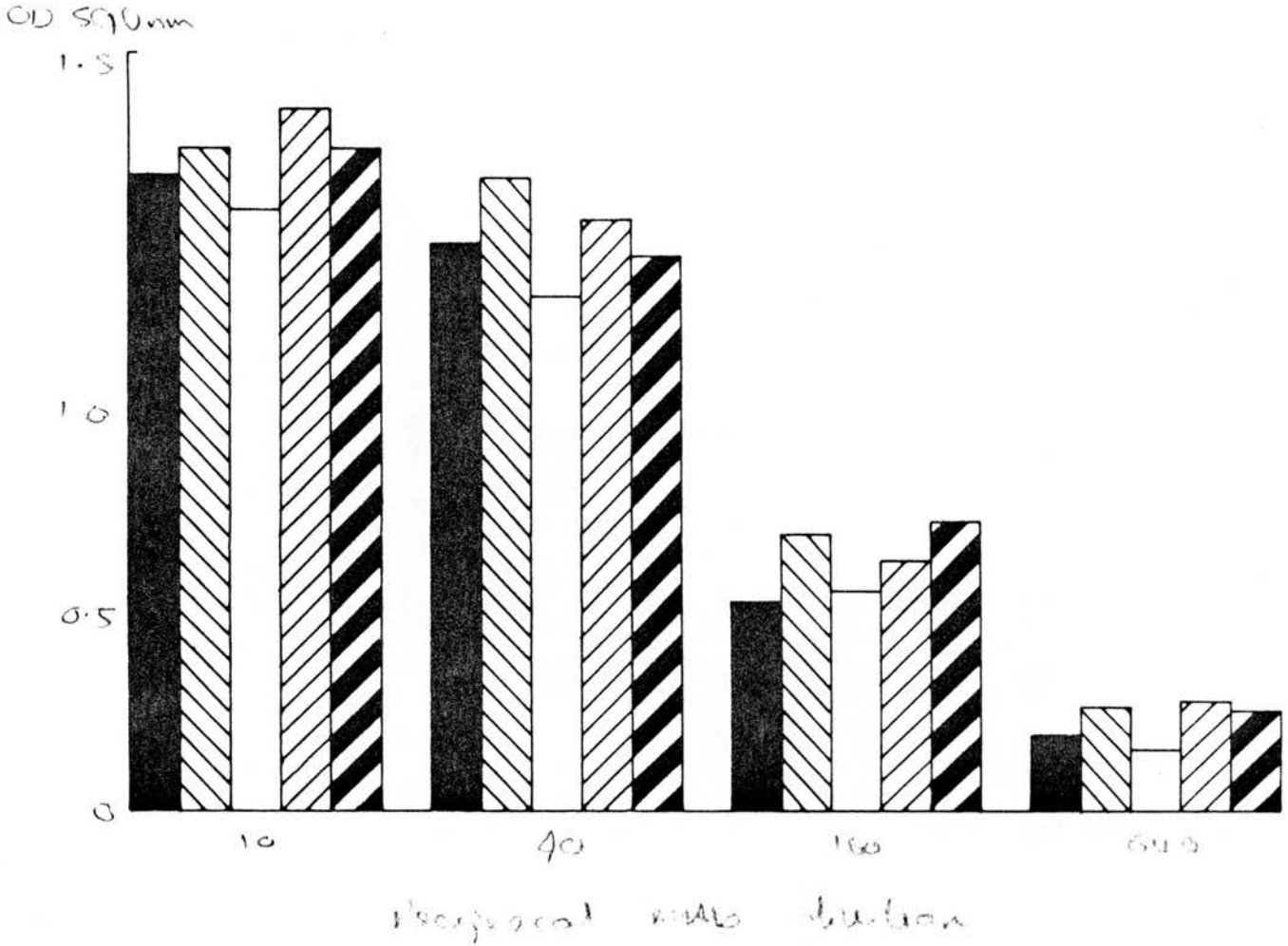
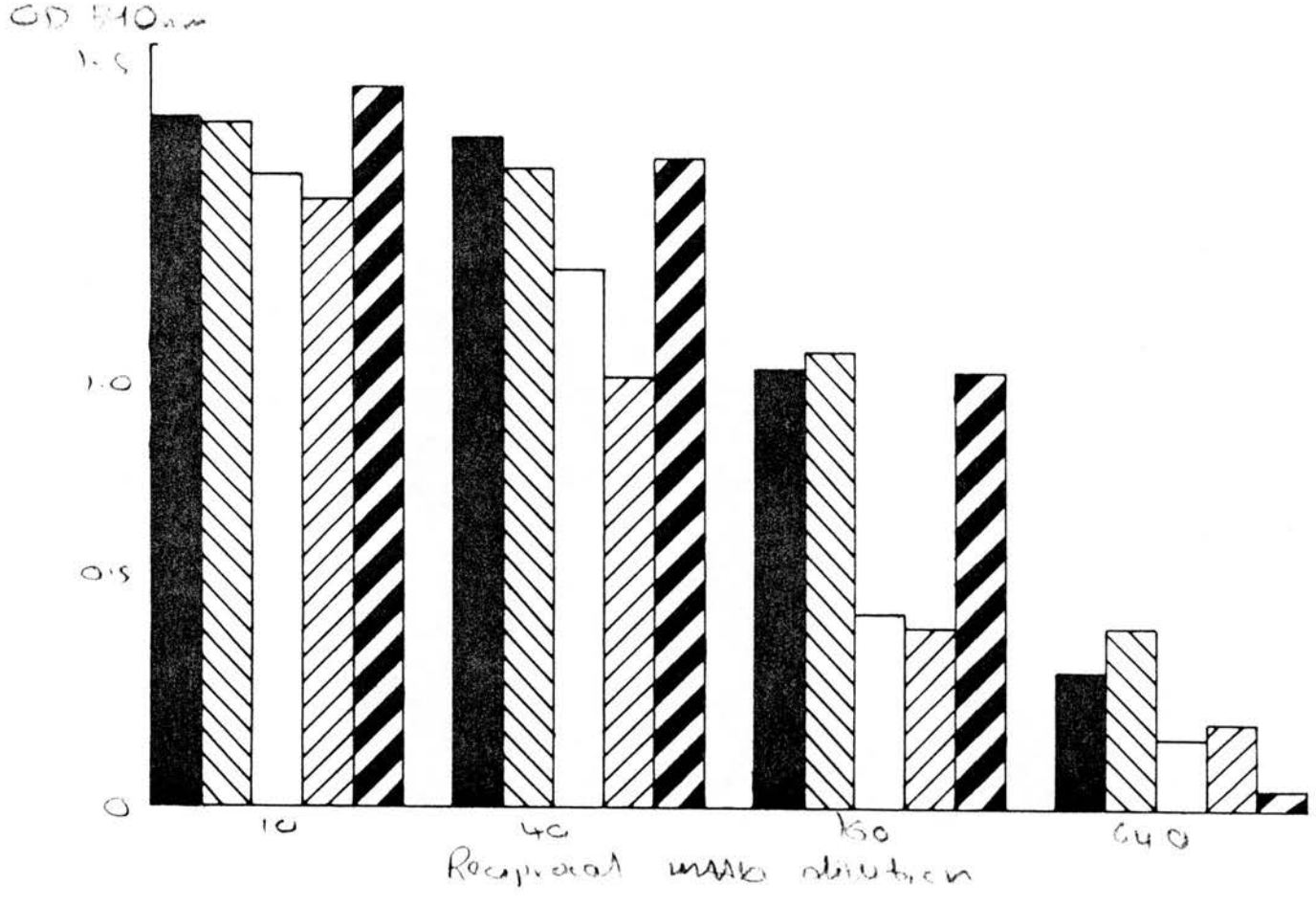


Fig 3b

fig 3c




OD 570nm
1·5
1·0
0·5
0
10
40
160
640
Reciprocal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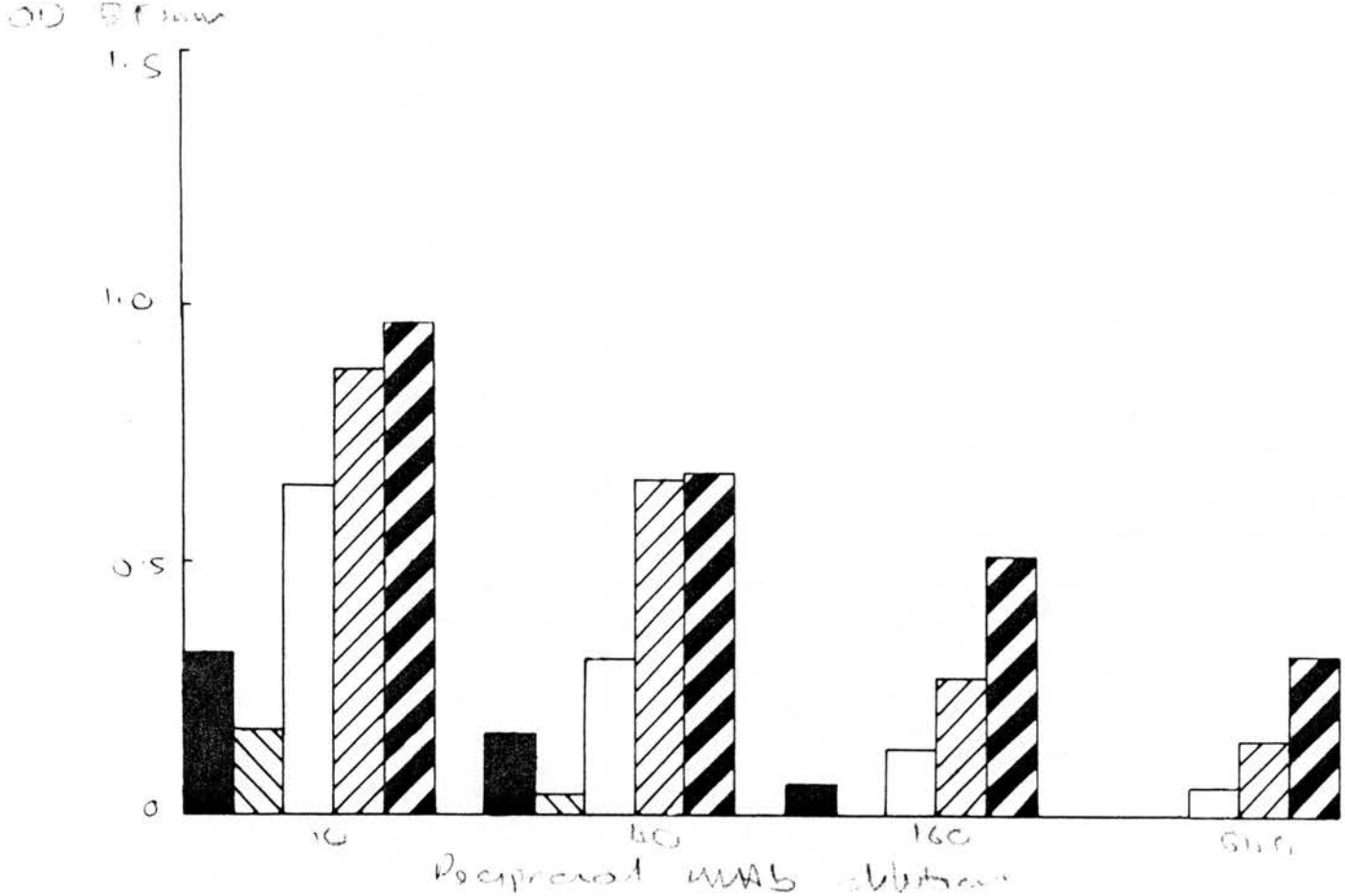
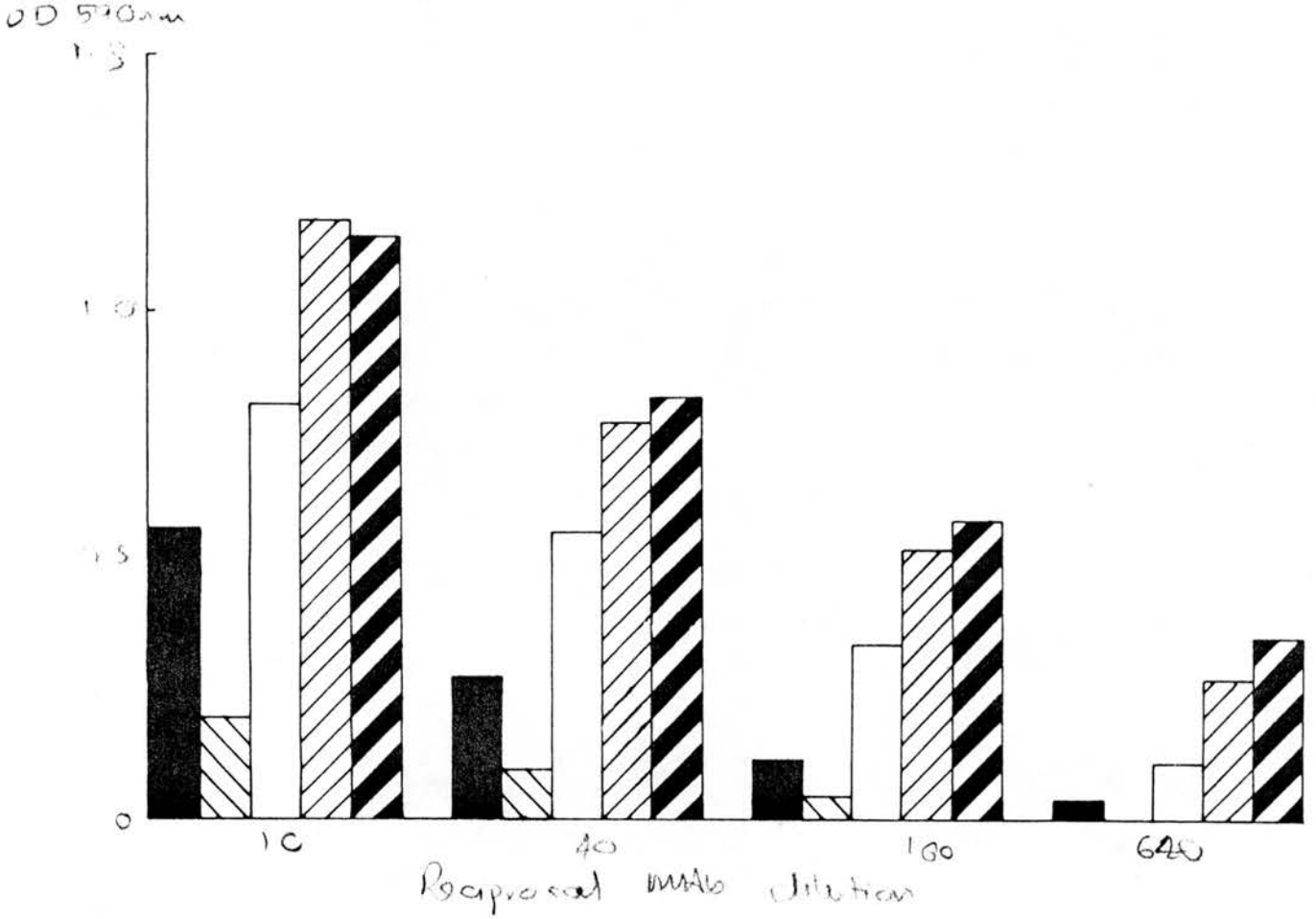
Reciprocal MMR dilution

4c

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160
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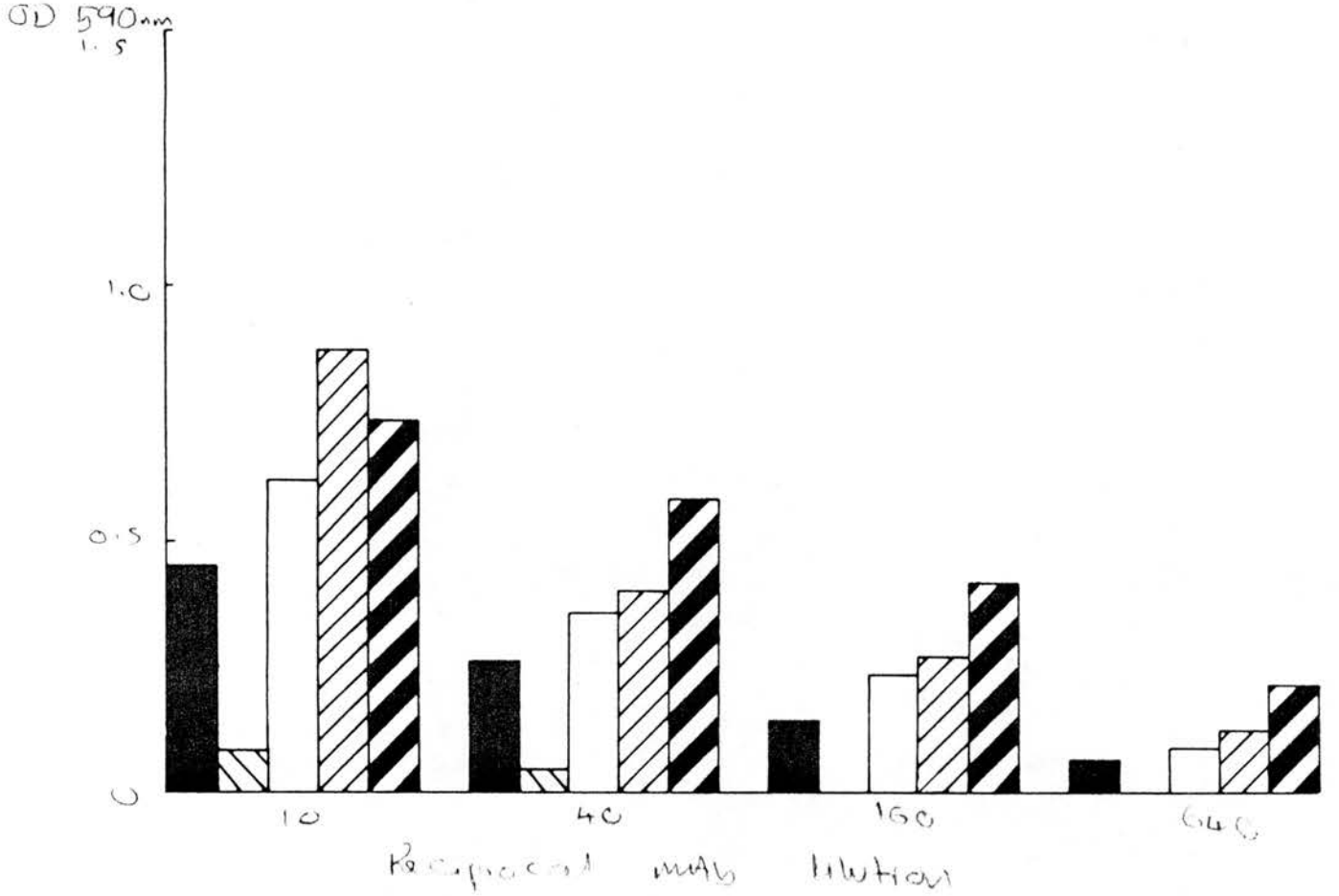
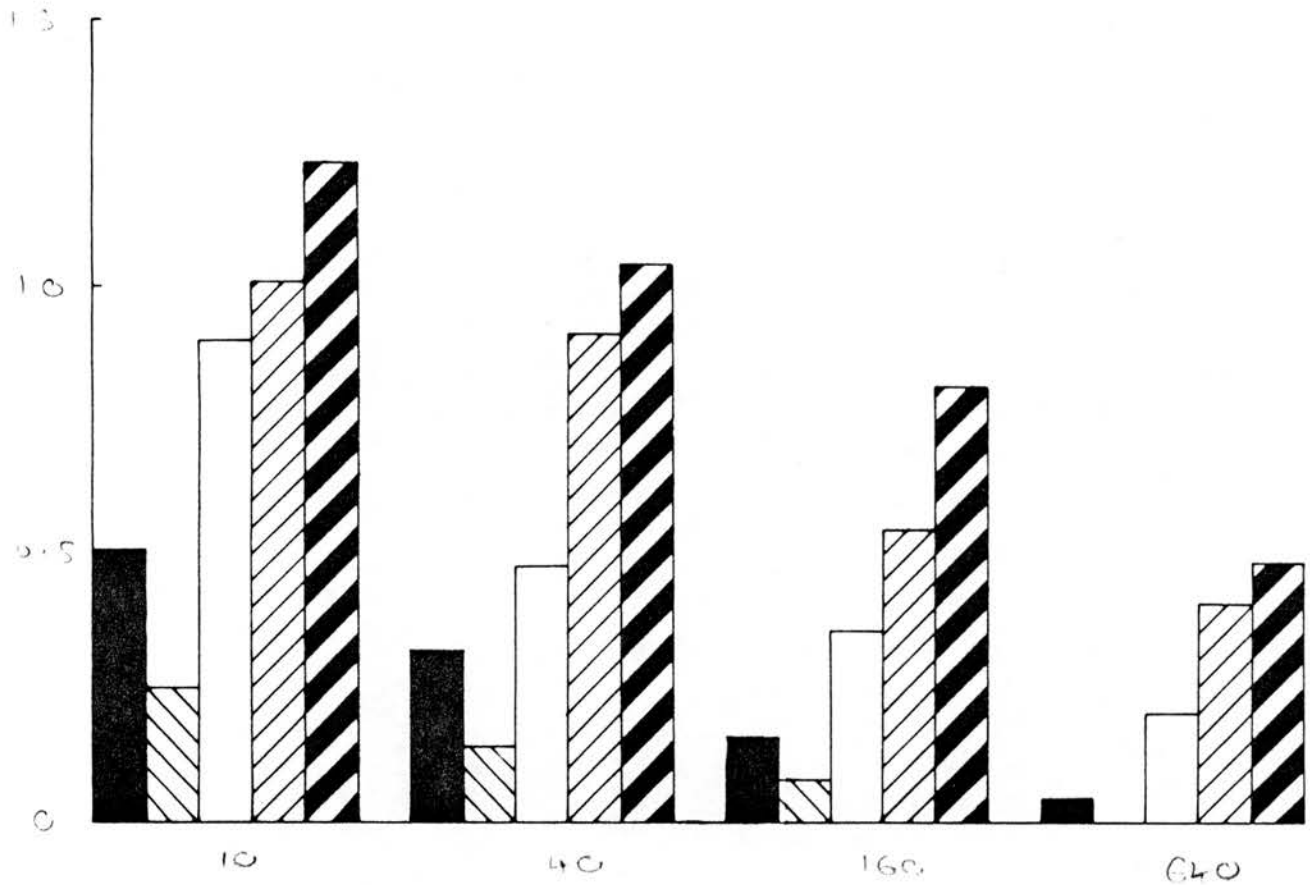
Reciprocal MAb dilution

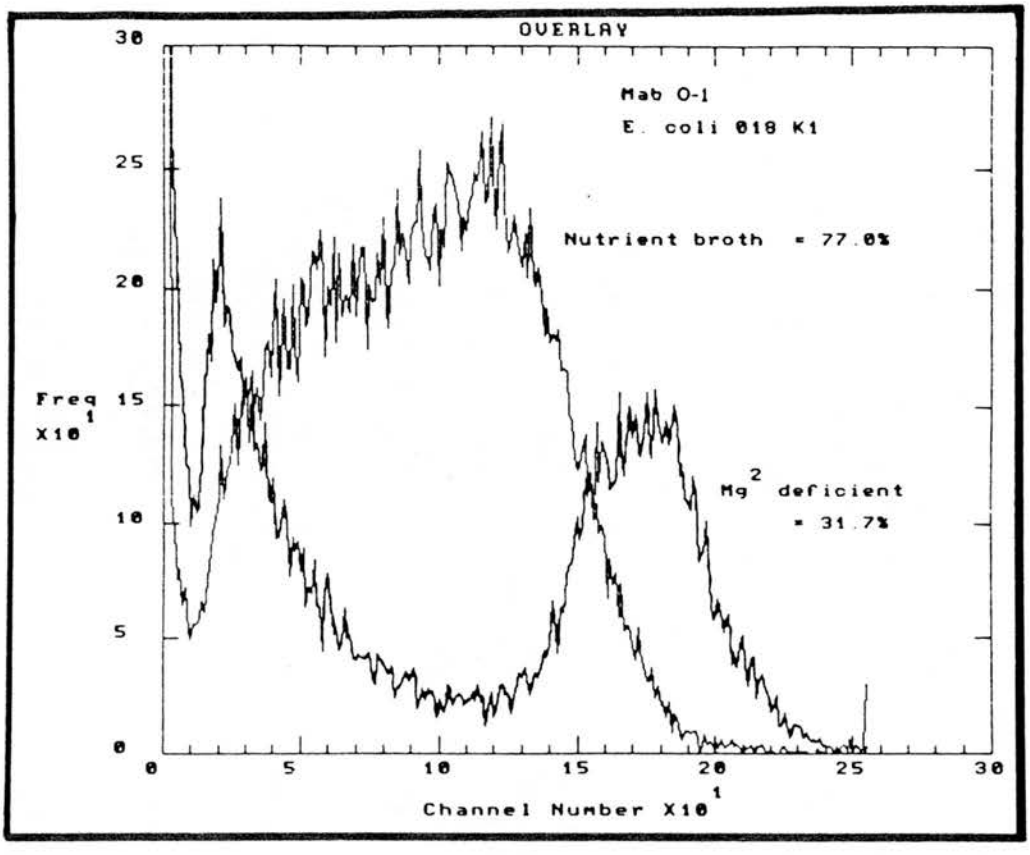

OD 590nm
1.5
1.0
0.5
0
10
40
160
640
Reciprocal mAb titration

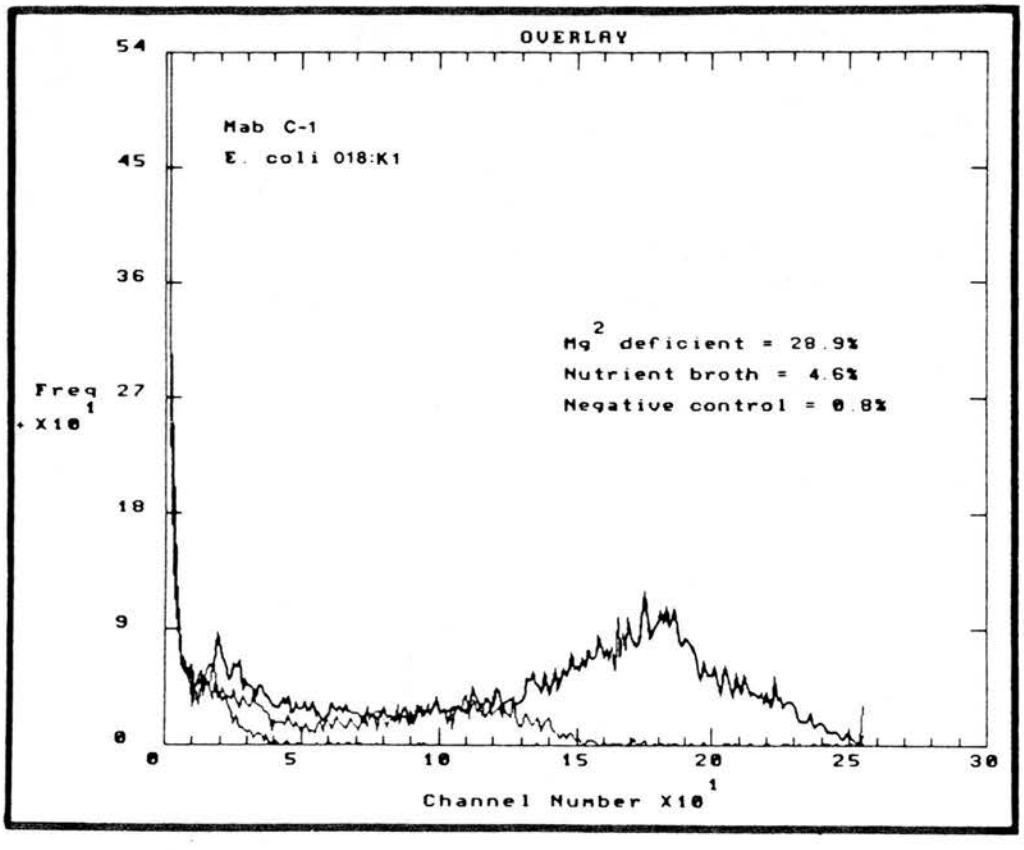
OD 510 nm



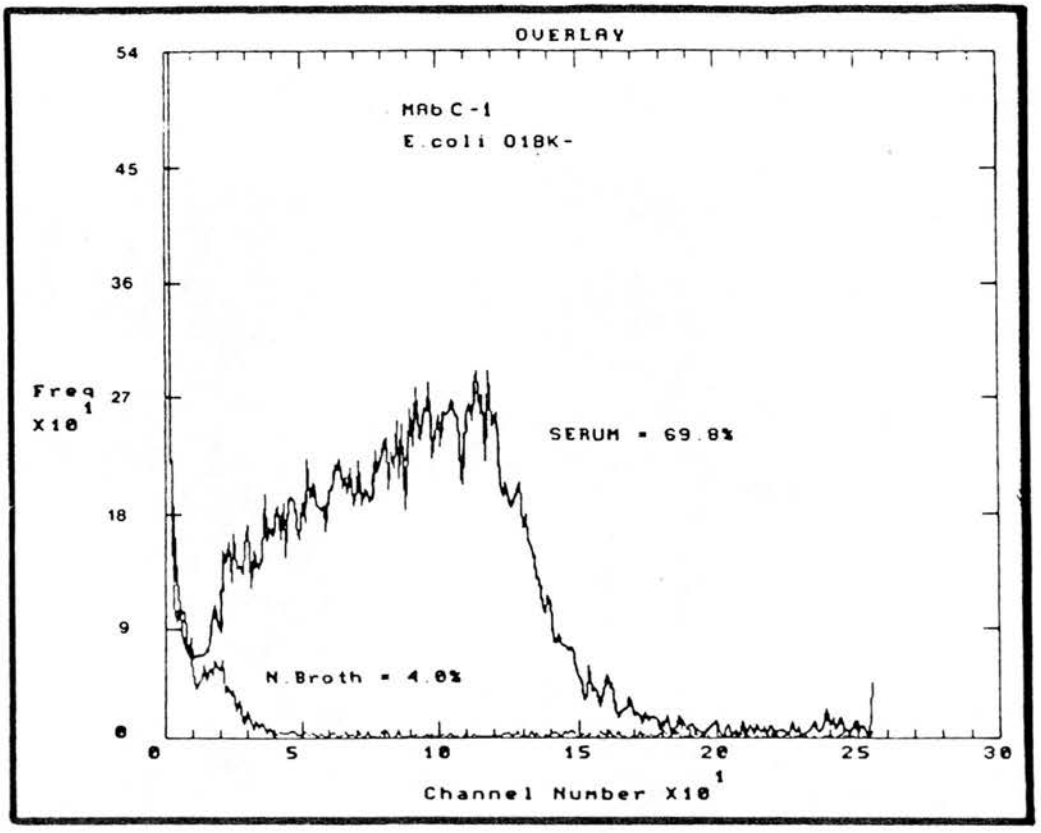
Reciprocal MAb dilution

Nelson / Bothgate + Foxton fig 5a + 5b





Nelson / Bathgate / Paxton figs 5C + 5D



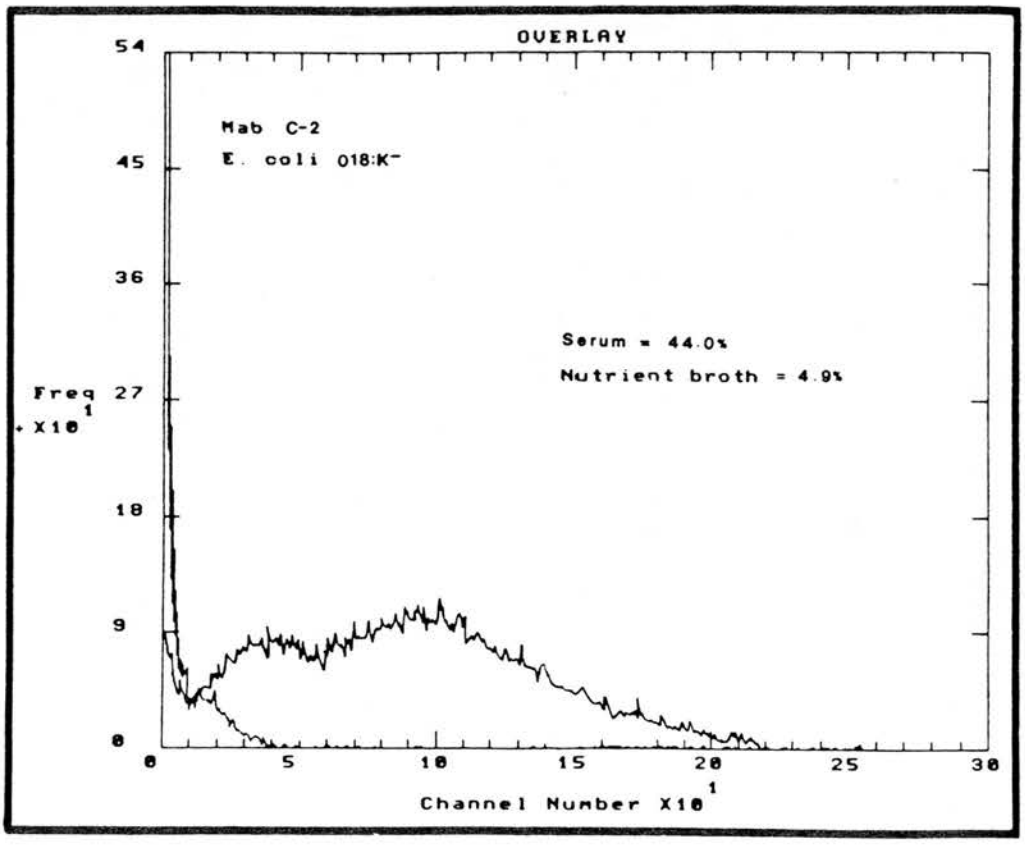


Table 1. Flow cytometric analysis of anti-LPS MAb binding to whole cells of four *E. coli* grown under different conditions. Percentage values represent the mean positive fluorescence of bacteria above background levels from three separate experiments. MAbs include an anti-O18, O-polysaccharide (O-1), and two anti-core MAbs (C-1 and C-2).

MAb	Growth medium	Percentage of cells showing positive fluorescence*			
		Escherichia coli strain			
		O18:K1	O18:K-	O18:Krf	O6:K5
O-1	Nutrient Broth	70(±19)	62(±14)	2(±1)	ND
	Nitrogen Deficient/ High Carbon	73(±10)	68(±7)	13(±6)	ND
	Iron-Depleted	74(±6)	50(±3)	3(±1)	ND
	Serum	68(±15)	67(±8)	8(±1)	ND
	Magnesium-Depleted	49(±9)	39(±9)	7(±0)	ND
C-1	Nutrient Broth	3(±1)	3(±2)	68(±11)	7(±1)
	Nitrogen Deficient/ High Carbon	2(±0)	2(±1)	56(±12)	1(±0)
	Iron-Depleted	10(±7)	7(±4)	66(±7)	7(±3)
	Serum	28(±16)	61(±11)	43(±14)	26(±12)
	Magnesium-Depleted	21(±4)	24(±5)	75(±13)	18(±8)
C-2	Nutrient Broth	2(±1)	6(±1)	26(±11)	3(±2)
	Nitrogen Deficient/ High Carbon	2(±0)	3(±1)	16(±6)	2(±1)
	Iron-Depleted	3(±2)	8(±3)	20(±5)	14(±9)
	Serum	16(±5)	34(±11)	20(±12)	32(±8)
	Magnesium-Depleted	12(±4)	18(±2)	28(±7)	10(±2)

* = mean ±SD ND = not done

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Chapter 4
Isolation and identification of bacterial antigens

I. R. POXTON & C. CAROLINE BLACKWELL

Isolation and purification of bacterial antigens have been invaluable in a number of areas. These techniques have been used in both human and veterinary medicine to help define the role of bacterial products and cellular components in the pathology of infectious diseases. Identification of antigens that elicit a protective immune response is necessary for development of effective vaccines. Many clinical diagnostic techniques are based on detection of bacterial antigens, and development of new methods continues, particularly those for organisms that are difficult to culture (brucellosis, legionnaires' disease) or for which there is no suitable artificial medium (syphilis), and for early detection of infective agents in paediatric medicine. Development of methods for screening or epidemiological studies of infectious diseases requires highly-purified, specific, antigens to reduce the possibility of cross-reactions with antigens of closely related but non-pathogenic species. Alterations in antigens following exposure to antibacterial agents can provide an insight into the mechanism by which the substance kills or inhibits bacteria. Comparison of similar antigens purified from closely related species can also be of use to taxonomists in their classification schemes.

There are two general categories of bacterial antigens: soluble products such as enzymes and toxins; and those antigens that form part of the cellular structure. Table 4.1 lists examples of these structural components in Gram-positive and Gram-negative bacteria and the mycobacteria. Fig. 4.1 is a diagrammatic representation of the surface layers of Gram-positive and Gram-negative bacteria.

The body of this chapter is organized into three main sections. The first section includes general considerations regarding growth of bacteria for antigen production and cell fractionation, and a review of the methods available for isolation of the different types of antigens. In the second section there is a review of methods available for identification of the different chemical classes of antigens. The last section contains specific recommendations for the application of these methods.

Methods for isolation of bacterial antigens

Growth of bacteria

General considerations

The conditions under which micro-organisms are grown markedly influence the production of extracellular products and their structural components. The essential pH, temperature, oxygen requirements, and nutritional characteristics must all be considered; therefore a review of the physiology of the organisms under investigation is highly recommended. Growth in a simple synthetic medium may result in a low yield of bacteria, or growth in different complex media may alter the composition of antigens [1,2,3,4]. There are some microbes such as *Mycobacterium leprae* for which there is no satisfactory artifical medium, and bacteria must be collected from human or animal tissues [5,6].

Production of extracellular proteins

It is often difficult to produce extracellular enzymes and toxins in laboratory media. Either yields are low or there are problems in purification due to the complex mixtures of protein digests and meat or yeast extracts required for their production. Several methods have been employed to overcome these problems. Hollander [7] reported a method for production of large quantities of staphylococcal toxins on solid media and Holme & Wadstrom [8] devised a continuous culture method for these toxins. Other methods include batch liquid culture with totally diffusible media and media in which complex growth requirements are added within a dialysis membrane to facilitate subsequent purification [9].

Table 4.1. Examples of structural bacterial antigens

	Gram-positive bacteria	Gram-negative bacteria	Mycobacteria
Proteins (including glycoproteins and lipoproteins)	Cytoplasmic membrane, capsular material, m proteins, fimbria-like protrusions, flagella, lectin-like molecules	Cytoplasmic membrane, pili/fimbriae, outer membrane proteins, Braun's lipoprotein, lectin-like molecules	Purified protein derivatives (PPD), proteins A, B, C & D protein antigens 5 & 6
Carbohydrates	Peptidoglycan, capsules and slime, teichoic acids, teichuronic acids and other secondary wall polymers	Peptidoglycan, capsules and slime	Peptidoglycan, mannan, arabinomannan, arabinogalactan
Lipocarbohydrates and glycolipids	Lipoteichoic acids	Lipopolysaccharide, enterobacterial common antigen	Mycosides, cord factor, wax D

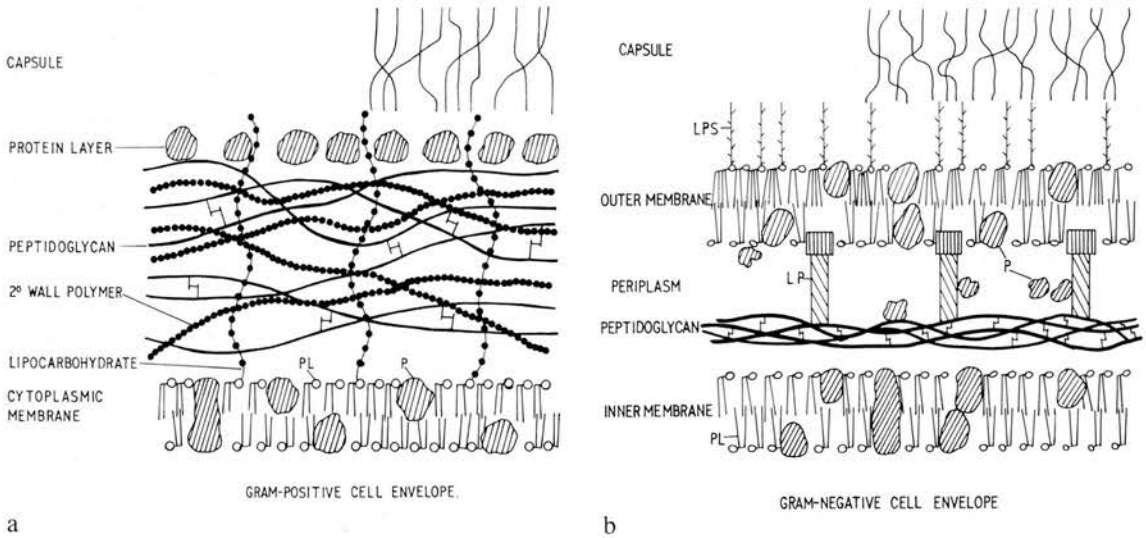

Fig. 4.1. Diagrammatic representation of the cell-surfaces of (a) Gram-positive and (b) Gram-negative bacteria. LP, lipoprotein; LPS, lipopolysaccharide; P, protein; PL; phospholipid.

Production of structural proteins

Culture on solid medium, liquid batch cultures, and continuous culture have been used successfully for growing large quantities of bacteria for analysis of structural components. The continuous culture method provides carefully controlled conditions so that the effects of nutrients [10] or dissolved oxygen [11] on components can be assessed. It may also be useful for investigating antigenic differences observed between actively growing and synchronized resting cells [12].

Radiolabelling of cells

Oeschger [13] has formulated a rich medium that supports rapid growth and efficient radiolabelling of proteins and nucleic acids that can be used for auxotrophic mutants. Addition of yeast extract treated with tyrosine carboxylase to a defined medium was used to prepare a rich medium from which at least 98% of the tyrosine was removed. Tyrosine is not usually an intermediate in any microbial metabolic pathway and the labelled tyrosine added to the medium is specifically incorporated into proteins.

Tyrosine is rapidly taken up and used by prototrophic strains of *Escherichia coli*. The enzyme tyrosine decarboxylase is active at mild conditions (pH 5.5) and has no requirements such as a metal cofactor. It is present in the medium in minute amounts and does not contribute significantly to its composition. Tyrosine is commercially available as a radiochemical with high specific activity.

Both radiolabelled uridine and thymidine can be used in this medium to label RNA and DNA. It is suggested that similar depletion of a specific component in a complex medium could be employed for the labelling of fastidious bacteria and those organisms for which there are as yet no defined media.

It is also possible to label the surfaces of bacteria with radioactive iodine. Exposed tyrosine or histidine residues may be iodinated with ^{125}I-lactoperoxidase. For an example of an application of this technique see Lambert & Booth [157].

Isolation of antigens

Introduction

The methods described below are, in the opinion of the authors, the most suitable that are currently available. There are, however, few defined recipes for the preparation of bacterial antigens. A technique that is well tried for one organism may not be satisfactory for another closely related microbe.

Most methods are concerned with releasing the antigen from the bacterial cell. Once this is released, the purification steps are similar to those for any biological molecule and standard biochemical separation techniques are employed. However, as a result of their special properties as antigens, affinity chromatographic techniques based on antibody–antigen binding (immunoadsorbent chromatography) are recommended whenever feasible.

Cell breakage

It is often desirable to disrupt the bacterial cell and fractionate sub-cellular particles before isolation of antigens. Fractionation of Gram-positive cells tends to be relatively straightforward as long as the presence of the sometimes highly active autolytic enzymes is acknowledged. Gram-negative bacteria are more difficult because of their more complex cell surface. Many different methods exist for cell disruption, but they can be conveniently divided into four types. Detailed accounts of these methods have been published [14,15]. Equipment and materials are listed in Table 4.2.

Table 4.2. Commercially available equipment and materials for cell breakage

Equipment/Material	Source*
French Pressure Cell	Aminco
X-Press	LKB Instruments
Braun Homogeniser	Braun Melsungen Int.
Lysozyme	Sigma Chemical Co.
Lysostaphin	Sigma Chemical Co.
Sodium dodecyl sulphate	BDH and Sigma
Glass beads	Sigma Chemical Co.

* See appendix for addresses.

Mechanical methods

These methods include grinding with alumina in a pestle and mortar, shaking with glass beads in a Braun homogenizer [16] or Mickel Disintegrator [17], passing the cells through a pressure cell, e.g. Hughes press [18], X-press [19] or French-press [20]. The grinding or shaking methods often result in extensive fragmentation of the walls and membranes. As a consequence, some particles are difficult to sediment by centrifugation. The advantage of the pressure cells is that the whole envelope remains largely intact.

Ultrasonic treatment

A variety of apparatus is available for this treatment. The resultant particles tend to be fragmented most by this method.

In the two methods above, much kinetic energy is given to the system, and efficient cooling is necessary. This can be achieved by using pre-cooled containers or placing the apparatus in ice/water or ice/ethanol mixtures. Also, giving the system short bursts of energy rather than continuous exposure is useful. The Braun homogenizer incorporates a CO_2 cooling system. Freezing, except in the cases of the Hughes and X-press, should be avoided so that agitation is not impaired. A general rule is that coccal-shaped bacteria tend to require much more energy for breakage than rod-shaped cells.

Enzymatic methods

These include naturally occurring autolytic enzymes which can be either purified and used specifically, or allowed to work without purification in a controlled manner. This latter method must be used with caution, as modification of antigens often occurs. For most purposes it is not recommended. Other enzymatic

methods include the use of the peptidoglycan solubilizing enzymes, lysozyme and lysostaphin. These treatments are recommended when protoplasts of Gram-positive or spheroplasts of Gram-negative bacteria are required, e.g. when preparing intracellular or membrane bound antigens. Protection against osmotic lysis must be maintained during the treatment. However, one of the major uses of such enzymes is not in antigen preparation, but in the preparation of DNA or other intracellular components or membranes for use in biosynthetic studies.

Chemical methods

These include detergents and chelating agents. Their action results in the destabilization of the membranes of the bacteria allowing release of membrane components. As the peptidoglycan layer tends to remain intact, there is no cell lysis; however, the combination of lysozyme and EDTA is useful for lysing Gram-negative cells. This method is described more fully below.

Fractionation of the cell envelope

Depending on the scale of the purification scheme, for economic reasons it is often necessary initially to employ crude fractionation and concentration steps followed by finer, often more expensive techniques. It is difficult to make generalizations; each preparation must be judged individually. Techniques, equipment, materials, and applications of methods for cell fractionation are listed in Table 4.3.

Method for Gram-positive bacteria

Once the bacteria have been broken open, the cell-envelope components can be fractionated. In Gram-positive organisms, this is relatively straightforward. The walls are collected from cells which have been broken, preferably by one of the methods listed under 'Mechanical methods', by centrifugation at 20 000 ***g*** for 15 min. The dense pellet of unbroken cells is easily distinguished from the lighter cell wall layer above. This wall layer is removed by careful washing with a jet of water or by gentle scraping. The autolytic enzymes must then be inactivated. It should be noted that this inactivation will also destroy any surface proteins present on the wall. A method for their preparation is given below (page 4.6). Inactivation of autolytic enzymes is accomplished by heating a suspension of walls to 80 °C for a few minutes. The method of choice is treatment of the suspension of walls at room temperature with an equal volume of boiling 4–5% (w/v) sodium dodecyl sulphate (or decyl sulphate), followed by stirring for several hours at room temperature [21]. This inactivates the autolytic enzymes and also solubilizes any non-covalently bound proteins, lipids or carbohydrates from the wall. The walls are washed free of sodium dodecyl sulphate with water by several cycles of centrifugation (20 000 ***g***) at room temperature. The sodium dodecyl sulphate is insoluble at low temperatures, but the decyl sulphate is very soluble and can be washed from the walls at low temperatures.

The cytoplasmic membrane of Gram-positive bacteria can be prepared in parallel to the walls if necessary. The bacteria are disrupted by mechanical methods, ultrasonic treatment or enzymatic methods. It is necessary to have the concentration of divalent cations (Mg^{2+} or Ca^{2+}) at 25 mM throughout the entire preparation in order that the integrity of the membranes is maintained. After the walls have been removed by centrifugation (20 000 ***g*** for 15 min), the membranes are pelleted from the supernate by centrifugation at 100 000 ***g*** for 1 h. The pellet consists of membranous vesicles of varying sizes.

Methods for Gram-negative bacteria

The fractionation of the envelope of Gram-negative bacteria is more complex than that of the Gram-positive. The separation of the rigid layer (peptidoglycan and associated molecules) makes use of the inert nature of this component. One method [22] involves treating whole cells sequentially with 0.03 M-NaOH, 0.4% SDS shaken with glass beads, 90% phenol, and finally more 0.4% SDS and glass beads. After extensive washing the peptidoglycan and covalently bound lipoprotein remain. If proteolytic enzymes are included in the preparation, pure peptidoglycan remains. Another method [23] uses disrupted cells which are treated with 2% SDS at 60 °C for 30 min. This leaves insoluble peptidoglycan, covalently linked lipoprotein and 'tightly associated' proteins which are not covalently linked (see page 4.6).

Membranes from Gram-negative bacteria can be prepared by two different approaches: one is based on cell disruption by treatment with EDTA and lysozyme; the other depends on the differential solubilization of the membrane in detergents.

The original method for separating inner and outer membranes of Gram-negative bacteria was developed by Miura & Mizushima [24] and later modified by Osborn *et al.* [25]. The first step involves the preparation of spheroplasts by treatment of log-phase bacteria with lysozyme and EDTA at 4 °C. The resulting spheroplasts are lysed osmotically or by ultrasonic treatment and the membranes are separated on a sucrose density gradient. The resultant bands

Table 4.3. Techniques used in fractionation and purification of bacterial antigens, including details of non-standard equipment and materials

Technique (C/F)[1]	Non-standard equipment, materials, suppliers[2]	Applications	References
Affinity chromatography (F)	Main supplier of supports—Pharmacia Lectins from Pharmacia and Miles	Any antigen if suitable ligand is available	124, 125
Ammonium sulphate precipitation (C)		Proteins (unbound or solubilized)	64, 65
Chemicals (chelating, chaotropic and dissociating agents, acids and alkalis) (C)		Outer membranes, non-covalently bound proteins, secondary cell wall polymers, fimbrial antigens	21, 27, 36, 37, 46, 48, 67
Chromatofocussing(TM) (F)	Pharmacia	Proteins	126
Detergent treatment (C)	Empigen BB from Albright and Wilson	Membrane solubilization, proteins, cell wall, and peptidoglycan preparations	29, 31, 32, 33, 38, 39
Gel filtration (C/F)	Main suppliers of gels; BioRad and Pharmacia	Proteins, LTA, ECA, secondary cell wall polymer	21, 59
Gradient centrifugation (C/F)	Ficoll™ and Percoll™ from Pharmacia	Membrane separation	24, 25, 26, 128
High-performance liquid chromatography (F)	Main suppliers LKB. Fast protein liquid chromatography (FPLC, Pharmacia)	Proteins	129
Homogenization (C)	e.g. Sorvall Omnimix (Dupont) MSE homogenizer Waring blender Vortex mixers	Exopolysaccharide, flagella, pili, fimbriae	42, 43, 61
Immunoadsorbent chromatography (F)	CNBr-activated Sepharose 4B (Pharmacia)	Any antigen if suitable antibody is available	45, 122, 130
Immunoprecipitation (F)	Insoluble matrices from Pharmacia	Any antigen if suitable antibody is available	130, 131
Ion-exchange chromatography (F)	Main suppliers: BioRad, Pharmacia and Whatman	Proteins, ECA, secondary cell wall polymers	21, 48, 54, 132
Isoelectric focussing (F)	Main suppliers of Ampholines: LKB and Pharmacia	Proteins	133, 134
Polyacrylamide gel electrophoresis (F)	—	Proteins, LPS	135, 136
Solvent extraction (C)	—	ECA, LPS, LTA, cell wall preparation	49, 50, 54, 59
Ultrafiltration (C)	Main suppliers: Amicon and Millipore	Rapid dialysis; concentration of specimens and column fractions; crude fractionation	9, 45
Ultrasonic treatment (C) (mild)	Sonic cleaning bath from Dawe Instruments	Outer membrane preparation	27, 28

[1] C = crude method, suitable for initial steps and large scale preparations.
F = fine, high resolution techniques, often expensive and difficult to use on large scale.
[2] See appendix for addresses of suppliers.

consist of a heavy band which contains lipopolysaccharide and is the outer membrane and a series of two or three lighter bands which are inner membranes. A further modification which is said to be more reproducible and can be used on a large scale is that of Yamato *et al.* [26]. After treatment of cells at high density with lysozyme and EDTA, they are broken by passage through a French pressure cell. The washed membranes are layered on to 44% (w/v) sucrose and ultracentrifuged. The inner membrane does not enter the sucrose and forms a band above it. The pellet is then layered on to a two-step sucrose gradient (52% and 56% w/v). The band that forms between the two concentrations is the outer membrane.

One of the authors (I.R. Poxton) has found that a simple and satisfactory method of preparing outer membranes from Gram-negative anaerobes is to subject bacteria to 10 mM-EDTA at pH 7.3 at 45 °C for 30 min. The suspension is treated for 60 s in an ultrasonic cleaning bath to detach the outer membranes. The cell bodies are removed by centrifugation (20 000 ***g*** for 10 min) leaving the outer membranes as vesicles in the supernate. The outer membranes can be recovered by ultracentrifugation, or concentrated by ultrafiltration or dialysis [27]. A similar method has been used by Hofstra & Dankert for a range of enterobacteria [28].

The other commonly used method for preparation of outer membrane proteins is based on that developed by Schnaitman [29]. A crude membrane fraction is prepared by EDTA/lysozyme or disruption of cells in a pressure cell. The membrane pellet is extracted with Triton X-100. The cytoplasmic membrane is solubilized leaving the outer membrane as the insoluble phase together with the peptidoglycan if the cells were disrupted without lysozyme. This method has been used widely for *E. coli* [30] and many other Gram-negative bacteria [31,32].

A similar method was developed using the detergent sodium lauryl sarcosinate (Sarkosyl) rather than Triton. This solubilized *E. coli* cytoplasmic membranes and allowed the outer membrane to be recovered by centrifugation [33]. It has been used successfully to prepare membranes of *Pseudomonas aeruginosa* (P.A. Lambert, personal communication) and *Bacteroides fragilis* (I.R. Poxton, unpublished results).

It has been noticed recently that outer membrane proteins of *Pseudomonas aeruginosa*, which have been prepared by detergent methods, are complexed with LPS even after separation on SDS-polyacrylamide gels. Some of the OM protein bands that stain with Coomassie Blue can also be visualized with monoclonal antibodies which are specific for LPS [161]. It is possible that this may be true for other Gram-negative bacterias.

Protein antigens

Free protein antigens

These consist of the antigenic extracellular proteins that are released into the culture medium during or after growth of bacteria. In the main they are enzymes, but some proteins have properties which interfere with biological processes of the host, e.g. neurotoxins and those that inhibit enzyme reactions. Extracellular proteins which have an adverse pharmacological effect on the host are often referred to as toxins or aggressins.

The isolation of extracellular proteins is complicated by the fact that they have to be purified from the culture medium which normally contains a complex mixture of protein digests and meat or yeast extracts (see 'Production of extracellular proteins', where methods and references for overcoming this problem are listed).

The first step in purification of an antigen from the culture fluid is almost always one of concentration. The two most commonly used methods are ammonium sulphate precipitation or ultrafiltration. If apparatus for ultrafiltration is not available, concentration in a dialysis bag and drawing out water and small molecules with Sephadex (Pharmacia), polyethylene glycol or by evaporation may also be used. Once concentrated, any of the standard biochemical techniques for protein purification can be used. To obtain a high degree of purity with minimum effort, one or a few of the most modern separation techniques for proteins should be used. These methods include affinity (or immunosorbent) chromatography, isoelectric focussing, Chromatofocussing (Pharmacia) or Fast Protein Liquid Chromatography (Pharmacia) (Table 4.3).

Bound protein antigens

Such proteins can be bound covalently or non-covalently to components of the bacterial cell. The former group include those covalently linked to peptidoglycan, e.g. Braun's lipoprotein [34] in the Gram-negative enterobacteria and the antigens A and B of *Streptococcus mutans* [35]. Both of these can be recovered from SDS-treated cell envelope preparations. The non-covalently bound proteins include those present on the outer surface of the wall of Gram-positive bacteria and in the membranes of both Gram-positive and Gram-negative bacteria. The former antigens can be removed by a variety of treatments, depending on how strongly they are bound to the surface. EDTA treatment (10 mM) of whole cells has proved useful for releasing weakly bound proteins [36] while 8 M-urea or 5 M-guanidine-HCl is required for removing proteins

forming regular arrays on detergent-treated envelope fractions of several Gram-positive organisms [37].

One of the major problems in isolating proteins from membranes is to solubilize them without causing denaturation. Various detergents have been tried. A general rule is that non-ionic detergents produce less denaturation than ionic detergents. Triton X-100 has been used extensively. The detergent Empigen BB has recently been used successfully for the isolation of gonococcal outer membrane protein I [38] and for an antigenically active protein from membranes of *Bordetella pertussis* [39]. This detergent appears to be particularly useful for the isolation of protein antigens which are easily susceptible to denaturation.

Another problem inherent in the purification of biologically active proteins (enzymes or toxins) is that inactivation often parallels purification. Associated molecules either in the culture fluid or structural components appear to exert a protective influence. Toxins often become toxoids, retaining their antigenicity but losing their toxic properties.

Carbohydrate antigens

Exopolysaccharides

This term includes any polysaccharide polymer that is found outside the wall of the Gram-positive bacteria or outside the outer membrane of the Gram-negative bacteria. The polymer can be a discrete capsule, of varying dimension, which can be visualized in the light microscope with India ink [40] or in the electron microscope by staining with Ruthenium Red [41]. It can also be found as an extracellular slime layer, which is readily sloughed off the surface into the surrounding medium.

The ease by which exopolysaccharides can be isolated and purified can vary greatly. The limiting factors tend to be how much polymer is produced and how firmly it is bound to the cell surface. The yield of exopolysaccharide, probably more than any other antigen, is greatly influenced by the conditions under which the bacteria are grown.

For reviews on exopolysaccharides see refs. 42 and 43. Brief guidelines will be given here for their production and isolation. To obtain maximum yield it is usually worthwhile to attempt to prolong the incubation time; many exopolysaccharides are produced in stationary phase. Usually a rich medium is better than a minimal medium; one with an increased carbon source and a reduced nitrogen source. Passaging the organism in the peritoneum of a mouse prior to culture is useful for selecting cells that produce exopolysaccharide, because they are less readily ingested by the peritoneal phagocytes. Growth on solid medium rather than liquid medium often results in higher yields of exopolysaccharide and also facilitates subsequent purification.

Isolation of exopolysaccharide involves harvesting the bacteria from solid medium by gently scraping the cells into a suspension of physiological saline containing 0.1% formaldehyde which fixes the cells and inhibits autolysis. The exopolysaccharide must then be removed from the cells. In the case of loosely attached slime, this step is almost unnecessary as most of it readily sloughs off. For more firmly attached capsular material, blending in a liquidizer or blender is required; the time depends on how firmly attached the polysaccharide is to the cell. Monitoring is done by the India ink method. Additional treatment with heat and dilute sodium hydroxide is sometimes suggested; but, if possible, their use should be avoided as subsequent purification is made more difficult due to the release of other macromolecules. The bacteria are removed from the blended suspension by centrifugation; high ***g*** forces ($> 10\,000$ ***g***) are often required due to the high viscosity of the suspension. The exopolysaccharide is precipitated from the supernate by the addition of four volumes of ice-cold acetone or ethanol. Depending on the nature of the polymer, complete precipitation may require 16 h. The precipitate is sometimes collected by winding it onto a glass rod; otherwise, low-speed centrifugation is required. The precipitate is usually washed several times in acetone, suspended in distilled water, and lyophilized. Further purification may be required and the reader should refer to the techniques discussed by Sutherland [43]. None of these techniques have proved generally satisfactory. The large relative molecular mass and the viscosity of the polysaccharides in solution hinders the development of satisfactory purification. Immunoabsorbent chromatography or immunoprecipitation of phenol-extracted capsule (see method for phenol extraction of lipopolysaccharide on page 4.8) are possibly the methods of choice, especially if an antiserum free of LPS antibodies or a monoclonal antibody preparation for the polysaccharide is available.

Teichoic acids (also teichuronic acids and other secondary cell wall polymers of Gram-positive bacteria)

These polymers are defined as the carbohydrates that are covalently linked to the peptidoglycan of the Gram-positive cell wall. Many of these polymers are true teichoic acids; they contain ribitol phosphate or glycerol phosphate. Analogous polymers are found in most, if not all, Gram-positive bacteria: the group-specific carbohydrate antigen of Lancefield group B streptococci [44], and the cross-reactive cell wall

antigen of several clostridia [45]. Traditionally such polymers were extracted by treatment of whole cells with cold 10% trichloroacetic acid (TCA), dilute NaOH, dilute *NN*-dimethylhydrazine, and several lytic enzymes [46]. Now that the nature of the linkage of the teichoic acid to peptidoglycan has been determined [47], it is probable that hydrolysis of cell walls with weak acid or alkali will result in the release of the polymer.

The method of choice for their isolation is as follows. Cell walls purified by SDS treatment (see page 4.4) at a concentration of 2–5% (w/v) are extracted with either acid (10% (w/v) TCA for 48 h at 4 °C) or alkali (0.5 M-NaOH 4 h at room temperature). The TCA is removed by six extractions with equal volumes of diethyl ether and the NaOH by de-salting on a column of Dowex 50 (NH_4^+ form) resin. Initially, extractions should be performed on a small scale to check that the polymer is not labile to either acid or alkali. Depending on the stability of the polymer one or other method is chosen for large scale preparation. Once extracted the polymers are purified by the methods described for TCA extracted pneumococcal C-carbohydrate [21] or NaOH-extracted teichoic acid of *Bacillus stearothermophilus* [48].

Peptidoglycan

The preparation of peptidoglycan from Gram-positive bacteria is performed by preparing pure cell walls by extensive SDS treatment, followed by solubilization of the secondary wall polymers as above. If the sole aim of the treatment is to prepare peptidoglycan, it does not matter which technique is used and it is preferable to use NaOH (0.5 M for 16 h at room temperature is probably sufficient). After extensive washing, pure peptidoglycan remains.

Preparation of peptidoglycan from Gram-negative bacteria has been described (page 4.4).

Lipocarbohydrates

Method for lipopolysaccharide (LPS)

The most widely used method for LPS preparation is based on the aqueous phenol extraction which was developed by Westphal, Luderitz & Bister [49].

1 Dried whole bacteria are suspended to an approximate concentration of 5% (w/v) in distilled water and heated to 68 °C. An equal volume of 90% (w/w) phenol also at 68 °C is added to the bacterial suspension. The mixture is stirred at 68 °C for 15–20 min, cooled in ice to aid phase separation, and centrifuged in glass or polypropylene tubes in a swing-out rotor for 15 min at a minimum of 3000 ***g***. The upper aqueous phase contains LPS, nucleic acids, and other polysaccharides. This solution is dialysed free of phenol against running water for at least 16 h at 4 °C. If any precipitate is produced, it is worth removing it at this stage by low-speed centrifugation (10 000 ***g***). The LPS is recovered from solution by concentrating the extract to about 10–20% of its original volume by rotary evaporation; this encourages micelle formation of the LPS. It is then collected by high-speed centrifugation (100 000 ***g*** for 3 h) as a gelatinous pellet. The pellet is washed in water by one or two cycles of high-speed centrifugation and then lyophilized. Although this is a standard method for LPS preparation, it should be noted that the product is often contaminated with nucleic acids and capsular polysaccharides. Further purification, preferably by immunoaffinity techniques, should be considered.

2 Some LPS molecules tend to be more hydrophobic than the classical *O*-specific LPS of enterobacteria for which the method above was developed. These hydrophobic molecules have a higher proportion of lipid or *N*-acetylated sugars and would partition into the phenol phase. Examples of such LPS molecules are those from rough (R) mutants of enterobacteria or some *Neisseria* spp. A method for the isolation of rough mutants was developed by Galanos *et al.* [50]. Whole dry bacteria are homogenized with 90% (w/v) phenol, chloroform, light petroleum (2:5:8) at 5–20 °C for 2 min. After removal of the bacteria by centrifugation in glass tubes (3000 ***g*** for 20 min), the chloroform and petroleum are removed from the solution by rotary evaporation and the LPS precipitated by dropwise addition of water. The product of this extraction is usually less contaminated with nucleic acid and capsular polysaccharide than the classic penol-water method. Glycolipids are, however, likely to be present.

3 A combination of the phenol-water and phenol-chloroform-petroleum methods has been used to obtain 'pure' LPS [51]. The crude dialysed aqueous phase obtained after initial phenol-water extraction was lyophilized and re-extracted with phenol-chloroform-petroleum as above. This produced a supposedly pure LPS. The technique requires further investigation and comparison with the preparation of the enterobacterial common antigen discussed below.

Enterobacterial common antigen (ECA)

Although the presence of an enterobacterial common antigen has been recognized since its discovery by Kunin in 1962 [52], it has been isolated only recently and its structure has been only partially determined. It appears to be a hydrophobic carbohydrate made up of a basic repeating unit of *N*-acetylglucosamine and

N-acetylmannosaminouronic acid with *O*-acetyl and possibly some fatty acid substituents [53]. It is included in this section on lipocarbohydrates because of its hydrophobic nature and because it often co-purifies with LPS when extracted by phenol-water.

1 From *Salmonella minnesota* by the method of Mannel & Mayer [54]. The aqueous phase from a phenol-water extraction of whole cells (see LPS above) is dialysed and lyophilized. It is then extracted with phenol, chloroform, and petroleum spirit as described for rough LPS. After the LPS has been precipitated from the phenol by the addition of water, the ECA remains in solution in the water-saturated phenol. The ECA is recovered after extensive dialysis, lyophilization, solubilization in water, and clarification by ultracentrifugation and chromatography on DEAE cellulose, eluting with 1.0 M-ammonium acetate in methanol.

2 From *Shigella sonnei*, ECA has been isolated by the method of Lugowski & Romanowska [55]. Whole cells are extensively disintegrated ultrasonically for 12 min in a buffer containing EDTA. Lysozyme is added and ultrasonic treatment is continued for 6 min. After stirring overnight at room temperature the suspension is centrifuged. The supernate is retained and the pellet re-extracted with water. Ethanol is added to both supernates to a final concentration of 85% by volume. Crude ECA is precipitated by the addition of acetone and is finally purified by silica gel chromatography and Sephadex LH20 chromatography (Pharmacia) with organic solvents as eluants.

Lipoteichoic acids (LTA) and other membrane associated lipocarbohydrates of Gram-positive bacteria

All Gram-positive bacteria appear to possess a membrane associated lipocarbohydrate. The true LTA is based on a poly-(glycerol phosphate) backbone with glycosyl and alanyl substituents [46]. Other lipocarbohydrates are known, e.g. the lipomannans of several *Micrococcus* spp. [56]. Although the lipid portion of the molecule is integrated into the cytoplasmic membrane, the carbohydrate is expressed outside the cell wall, both as an antigen [57] and as a component involved in adherence to surfaces [58].

An extraction technique similar to the isolation of LPS has been developed and evaluated by Coley *et al.* [59]. Cells which have been defatted by treatment with chloroform-methanol (2:1) overnight are extracted with 40% (w/w) aqueous phenol at 4 °C or 60 °C. The aqueous fraction is purified by nuclease treatment and fractionation on Sepharose 6B (Pharmacia). The LTA elutes from the column in the void volume. The conclusion from this study [59] was that there was no single general method available and that the polymer was still contaminated with nucleic acids. However, we have found that the membrane lipocarbohydrate from such diverse organisms as *Clostridium difficile* and Group B streptococcus can be isolated by the cold phenol method [59] followed by treatment with DNase and RNase at pH 5.0 in acetate buffer, partial fractionation on Sepharose 6B, and final purification on an immunoabsorbent column [45,60].

Appendages

Flagella

Parton's method for isolation of flagella from *Salmonella minnesota* is given here as an example of this technique. Sixteen-hour broth cultures (Oxoid Nutrient Broth no. 2) grown at 37 °C were collected by centrifugation at 4 °C, washed once in saline, and resedimented. Growth at 44 °C prevents production of flagella. Cells from one litre were resuspended in 50 ml cold saline and blended in an MSE homogenizer at full speed for 2 min with the flask cooled in an ice water bath. The sheared cells were removed by centrifugation at 10 000 ***g*** for 30 min at 4 °C. Flagella were concentrated from the supernate by dialysis against polyethylene glycol 4000 (British Drug House) or collected by centrifugation at 100 000 ***g*** for one hour [61].

Further purified preparations can be obtained by enzyme digestion of the flagella with trypsin and ribonuclease followed by equilibrium density centrifugation [62].

Pili or fimbriae

Isolation of pili from *Neisseria gonorrhoeae* is given here as an example. Organisms were grown on trays of clear typing medium (GC Medium, Difco) at 36 °C in an atmosphere of 5% (v/v) CO_2. Colony types 1 and 2 are pilate but colony types 3 and 4 are not. A plate of clear typing medium inoculated at the same time as the trays can be examined by means of a stereomicroscope with a double system of substage lighting [63] for the presence of pilate colony types. Gonococci were harvested into ice-cold ethanolamine/HCl (0.15 M, pH 10.5) and subjected to mild shearing in an Ultra-Turrax homogenizer (Vortex Mixers, Hampton, Middlesex) for 2 min. The organisms were removed by centrifugation at 23 000 ***g*** for 30 min. The pili were precipitated from the supernate by adding saturated ammonium sulphate (made alkaline by the addition of 0.1 volume of tenfold concentrated ethanolamine buffer) to give a final saturation of 10%. Pili were recovered by centrifugation at 10 000 ***g*** for 1 h and purified by further cycles of repeated disaggregation in ethanolamine buffer and precipitation with

ammonium sulphate to remove contaminants of outer membrane bleb material. Pili were finally washed in 2 M-NaCl and stored at 4 °C in 1 M-NaCl containing 8 mM-sodium azide.

The purity of the preparation was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on a separating gel of a linear 10–25% acrylamide gradient [64,65]. Gels were deliberately overloaded and no contaminant protein bands were detected on staining with Coomassie Brilliant Blue R250.

A number of fimbrial antigens have been associated with pathogenesis of intestinal diseases caused by *E. coli*: colonization factors, CFA I and CFA II, in humans; K88 in piglets; and K99 in calves. Production of these antigens is temperature dependent. Cells grown below 18 °C do not express the fimbriae and these have been used to produce specific antisera against the fimbrial antigen by absorbing antisera produced against the same strain grown at a higher temperature. Lysine in the growth medium represses the production of K99 and a minimal casein medium, 'minca' medium, is recommended [66]. Minca medium is composed of: KH_2PO_4, 1.36 g; $Na_2HPO_4 \cdot 2H_2O$, 10.1 g; glucose, 1 g; trace salts solution 1 ml; casamino acids (Difco) 1 g; agar (Difco) 12 g; distilled water, 1000 ml. Trace salts solution contained: $MgSO_4 \cdot 7H_2O$, 10 g; $MnCl_2 \cdot 4H_2O$, 1 g; $FeCl_3 \cdot 6H_2O$, 0.135 g; $CaCl_2 \cdot 2H_2O$, 0.4 g; distilled water 1000 ml.

Extraction and purification of each of these antigens has been described by a number of groups. Methods for extraction are listed in Table 4.4. Altmann *et al.* [67] report that all three antigens—K99, K88, and CFA—as well as pili from *Bacteroides (Fusiformis) nodosus* (the bacterium causing foot rot in sheep) were extractable by their KSCN method.

Purification usually involves concentration of the extracted pili by acid or ammonium sulphate precipitation, followed by fractionation on Sephadex columns. Purity is assessed by SDS-PAGE and isoelectric focussing. Table 4.5 lists three fimbrial antigens, the purposes for which they have been isolated, and the reference to the specific method.

Table 4.4. Extraction methods for fimbrial antigens

Antigen	Extraction method	Reference
CFA	3 M-KSCN, pH 7.1;	67
	heating 60 °C, 20 min, followed by homogenization	68
	Homogenization in Waring blender, 4 °C, 4 min	69
K88	3 M-KSCN, pH 7.1;	67
	shearing in Virtis mixer 30 min in ice	70
K99	Heating broth culture 60°C;	71
	homogenization;	72
	3 M-KSCN, pH 7.1	67

Table 4.5. Purposes for which fimbrial antigens have been isolated

Antigen	Use	Reference
CFA	Production of agglutinating antibody	69
	Characterization of amino-acid sequence and relative molecular mass of pili	68
	Serological properties and chemical purification	73
K88	Determination of amino-acid composition	70
K99	Chemical purification and amino-acid analysis	67
	Structure-function relationship of fimbrial proteins	74
	Comparison of acid and ammonium sulphate preparation methods and haemagglutinating properties of pili	75
	Heterogeneity of K99 antigens	76, 77

Mycobacterial antigens

Janicki and co-workers from several independent laboratories devised a system of identification and nomenclature for mycobacterial antigens. The system employs immunoelectrophoresis and polyacrylamide gel electrophoresis and utilizes small quantities of readily available reagents. The immunoelectrophoresis reference reagents and nomenclature are sometimes called United States-Japan reagents because their production was sponsored by the United States-Japan Co-operative Medical Sciences Program through the National Institutes of Allergy and Infectious Diseases. They have been used by many investigators to identify their products and/or to relate their methodologies to a common reference point. Arabic numeral designations of antigens refer to the nomenclature of Janicki *et al.* [78]. Table 4.6 relates the early fractions described by Seibert [79] to the United States-Japan system.

There is no standard starting material for the isolation of these antigens. Sterile filtrates of old, autolysed cultures are available in large quantities and these have been used for antigen preparation. Whole cells or cell walls extracted to decrease their lipid

Table 4.6. Mycobacterial antigens—US-Japan Nomenclature

US-Japan nomenclature	Seibert's fraction
1, 2	Polysaccharide I
3	Polysaccharide II
1, 2, 4, 5, 6	Protein A
1, 2, 5, 6, 7	Protein B
2, 6, 7	Protein C

Table 4.7. Growth factors affecting composition of mycobacterial antigens

Age	[80, 81, 82, 83, 84, 85, 86]
pH and medium	[87, 88]
Temperature	[83]

content are used for preparations from which cell wall polysaccharides are to be recovered. Disruption of the cells can be accomplished by grinding, pressure or sonication; few antigenic differences in these cell extracts could be detected [79]. Variations in growth conditions result in differences in the antigenic composition of culture filtrates. These are mainly quantitative rather than qualitative differences (Table 4.7).

The reader is referred to the excellent review of Daniel & Janicki [78] for details of purification and isolation of mycobacterial antigens. Methods for purification of a number of these are listed in Table 4.8.

Ribosomal vaccines

The efficacy of early ribosomal vaccines was attributed to the double-stranded RNA they contained, but subsequent studies showed that they contained immunogenic amounts of cell surface antigens. At least part of their protective effects has been ascribed to these contaminants [108,109]. The mechanisms by which these preparations induce a protective immune response are not yet clear [110]. Three hypotheses have been suggested: (1) all protection is due to contamination with cell surface antigens; (2) ribosomal RNA induces protective immunity in an as yet unidentified manner; (3) the ribosomes act as adjuvant for cell surface antigens [111].

Studies on ribosomal vaccines have had two purposes: to prepare a protective vaccine; and to identify the protective component. The second aim is important in that if the first hypothesis is true, energies would be better used in isolation of antigenic surface components. The use of genetically defined strains of organisms presents opportunities to prepare ribosomal vaccines from which certain antigens can be eliminated [108].

Monomeric ribosomes are usually required for vaccines. Maintaining the isolated ribosomes in the appropriate solution is vital for preventing their dissociation or disruption into small subunits or fragments. Purification of the monomers requires balancing the removal of adsorbed or absorbed components with maintaining the integrity of the monomeric ribosomes.

Disruption of cells by chemical lysis provides less opportunity for cell wall components to adsorb to the ribosomes. Chemical lysis of *Pasturella multocida* cells resulted in less LPS contamination of ribosomes than mechanical disruption [112]. Lysis with specific enzymes, such as the phage-associated lysin for specific disruption of *Streptococcus pyogenes* [113], may be effective in eliminating particular cell wall components.

Procedures for isolation of ribosomes for immunogenic purposes are basically identical to those for preparations used for biochemical and biophysical analysis. A number of methods have been used for the isolation of ribosomes and the removal of contaminating material. Differential centrifugation was used in early studies to preferentially remove the ribosomes from the cellular extract [114,115]. Gel filtration and electrostatic disruption were used as gentle methods for purifying and maintaining the integrity of the ribosomes [116,117,118,119].

A number of techniques have been used to remove contaminating antigens from the ribosomes. Electrophoretic methods have not been successful because many of the undesired antigens have net charges similar to those of the ribosomes. Treatment with specific enzymes, such as a phage-associated glycanase, has been used to degrade and remove capsular carbohydrates from ribosomal preparations of *Klebsiella pneumoniae*. Of particular value are techniques of affinity chromatography using specific ligands for adsorption of minute quantities of antigens. Ribosomes are eluted over columns to which monoclonal antibodies to the contaminating antigen have been

Table 4.8. Selected major purification method for mycobacterial antigens

Antigen(s)	Method	Reference
Preparation of PPD; PPDs from mycobacteria other than *M. tuberculosis*	Acid precipitation, ammonium sulphate precipitation	89
Proteins A, B, C, and D and polysaccharides I and II, none highly purified	Ethanol-acetic acid precipitation	79
Highly purified antigenic arabinogalactan and arabinomannan; non-antigenic mannan and glucan	Alkaline extraction, ethanol precipitation, ion-exchange chromatography	90, 91
Protein- and polysaccharide-rich fractions, none highly purified	Paper curtain zonal electrophoresis	92
Protein- and polysaccharide-rich antigenic fractions, none highly purified	DEAE-cellulose ion-exchange chromatography	93
Protein- and polysaccharide-rich antigenic fractions, none highly purified	Acid solubility, molecular exclusion chromatography	94
Purified tuberculin-active peptide	Hydrochloric acid extraction and picric acid precipitation, gel filtration, ion-exchange chromatography	95, 96
Highly purified antigenic proteins, low yields	Serial ammonium sulphate precipitation, gel filtration, ion-exchange chromatography, zonal electrophoresis	82, 97, 98, 99, 100
Small quantities of highly purified antigenic proteins	Acrylamide gel electrophoresis	101, 102
Antigenic arabinogalactan and arabinomannan	Concanavalin A affinity chromatography	103
Antigenic proteins. Highly purified antigen 5	Immunoabsorbent affinity chromatography	104, 105, 106
Glycolipid antigen		107

coupled. This has been successfully applied to the removal of LPS from *P. multocida* ribosomes [120]. It has also been suggested that new methods may be developed in which lectins are successfully used as the immobilized ligand to remove carbohydrate containing components.

Methods for determining the chemical content of ribosomes with particular reference to analysis of cell wall and membrane contaminants are described by Gregory & Schechmeister [121]. Phillips & Rimler [120] have also described a formaldehyde fixing procedure for the stabilization of ribosomal preparations that also enhanced their role as immunomodulators.

The most recent recommendations for the preparation of ribosomal vaccines are those that minimize contamination of the ribosomes by cell wall or membrane antigens (chemical rather than mechanical disruption) and removal of undesired components by affinity chromatography procedures with specific monoclonal antibodies. The limitations of these procedures lie in providing conditions for affinity reactions to occur so that unwanted antigens are removed but the integrity of the ribosomes is preserved.

Methods for the identification of bacterial antigens

This section is restricted to methods that may be used for the identification and quantification of antigens during and after their preparation. The methods used for routine detection of free or bacteria-bound antigens in the diagnosis of bacterial infections or in epidemiological investigations by reference laboratories are excluded. Details of these methods are found in Volume IV.

Review of methods and their applications

A great variety of methods are available for the identification of bacterial antigens. Early methods included immunoprecipitation in gels and tubes, agglutination and haemagglutination tests, complement fixation tests, and simple forms of immunoelectro-

Table 4.9. Methods for identification of bacterial antigens and examples of applications

Method	General applications	References (general)	References (specific examples)
Immunoelectrophoresis for precipitating antigens		131, 137, 138, 139,	45, 140, 158, 159
(1) Crossed immunoelectrophoresis	High resolution method for detecting precipitating antigens; quantification of antigens in complex mixtures Intermediate gels used for detecting cross-reacting antigens or antibodies		
(2) Rocket immunoelectrophoresis	Initial screening for precipitating antigens Screening column fractions Quantification of pure antigens		
Enzyme-linked immunosorbent assay (ELISA)	Monitoring antibody response in experimental animals and antibody production by hybridomas; highly sensitive technique for detecting antigens; quantitative inhibition of ELISA useful for detecting cross-reactive antigens and showing how purified antigens contribute to a complex reaction (see Fig. 4.3)	142, 143, 144	51, 145, 146, 147
Polyacrylamide gel immuno methods		131, 148, 149, 150	149, 151, 152, 153, 154, 160
(1) Direct immunodetection on gel (SGIP and GIRA)	Suitable for any antigen that can be separated on PAGE; extremely sensitive; both types of technique rely on SDS removal prior to immunoassay; slab gels useful for screening large numbers of samples; becoming increasingly used for monoclonal antibody studies		
(2) Electro-transfer/blotting methods	Monitoring purification procedures Electro-transfer methods have largely superseded direct methods		

[1] SGIP = SDS gel immunoperoxidase; GIRA = gel immunoradioassay.

phoresis. It is questionable whether any of these tests still have a place today. Although often sensitive and quantitative, their inherent lack of specificity and low resolving power make them largely unsuitable. There are several reports which show that antigen preparations once thought to be pure are in fact complexes of antigenic and non-antigenic molecules. It is only when high-resolution separation techniques are used in their preparation and subsequent analysis that these problems become apparent; see refs. cited by Wadstrom [9].

The range of immunological techniques that are most applicable to the identification of bacterial antigens are listed in Table 4.9. These methods have been divided into three basic types: immunoelectrophoretic methods for precipitating antigens; indirect ELISA methods for antigens that can be bound to polystyrene or similar material; and immunodetection of antigens that have been separated on polyacrylamide gels. Examples of these are shown in Figs. 4.2–4.5.

It should be stressed that immunological methods should not be used alone. Other sensitive analytical techniques should be included so that non-antigenic molecules can be detected. The various forms of polyacrylamide gel electrophoresis and isoelectric focussing are ideal for these purposes. It is important that gels are deliberately overloaded so that minor contaminants may be detected. The highly sensitive silver stain which can be applied to both proteins and carbohydrates is especially useful [122,123].

Recommendations

Purpose for which antigen is prepared

Before starting to prepare an antigen, careful consideration must be given to the purpose for which it is being isolated. If it is to be used as a vaccine, care must be taken to ensure that its native immunogenic properties are retained. Extensive fractionation is often unnecessary. If, however, an analysis is to be made to determine the chemical composition or immunologic specificity, ultimate purity is necessary. Although antigenicity

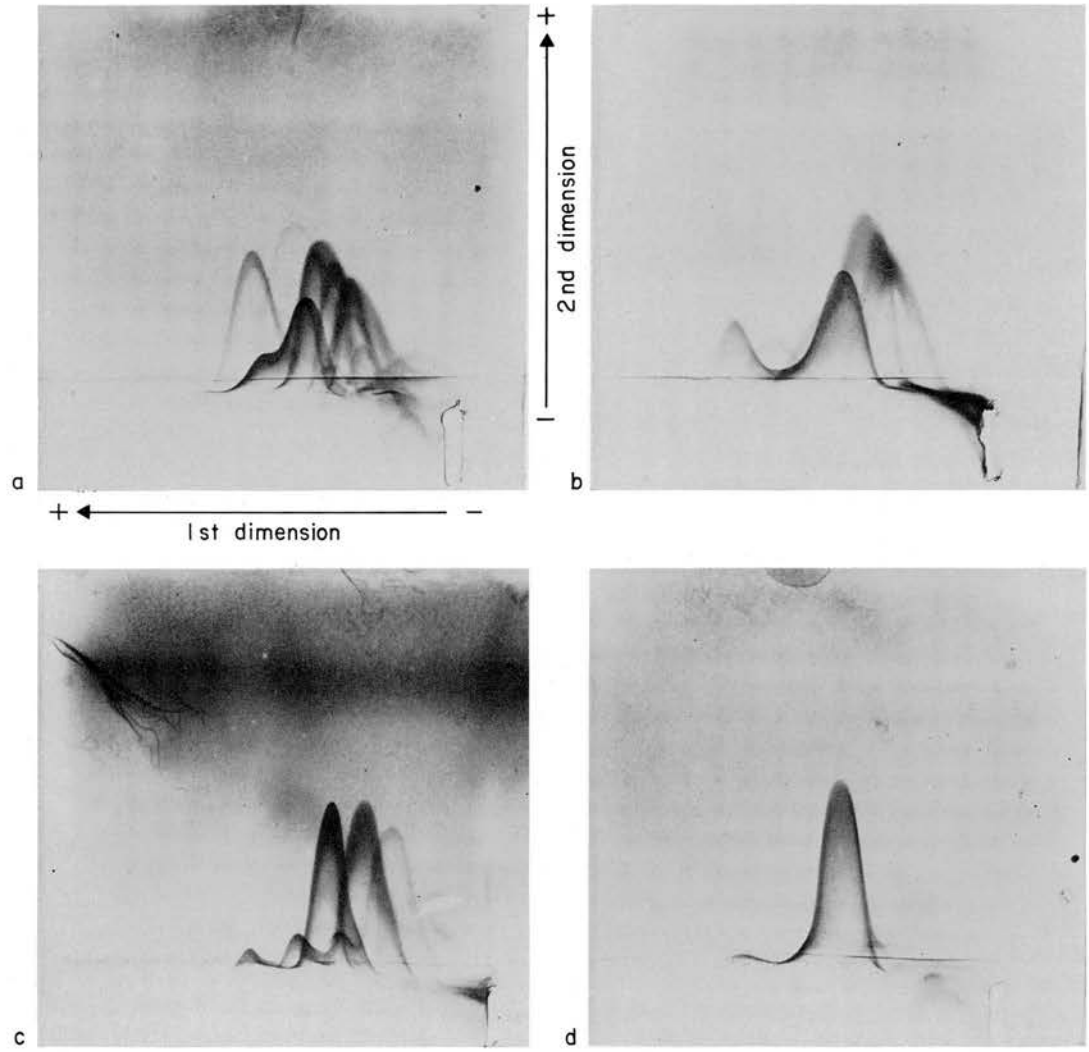

Fig. 4.2. Crossed immunoelectrophoresis of *Bacteroides fragilis* antigens. (a) EDTA-extracted outer membrane (OM) complex of a reference strain (20 μg protein in 10 μl) was run into the first dimension for 1.5 h at 12.5 V cm^{-1}, and into the second dimension, containing homologous antiserum raised to whole cells (0.25 ml antiserum in 3.5 ml agarose), for 16 h at 12 V cm^{-1}. The gel was stained by Coomassie Blue. (b) The same method as (a) was used but the OM complex was heated to 121 °C for 15 min before running in the first dimension. (c) A 45% (w/v) aqueous phenol extract of whole cells of the *B. fragilis* reference strain (50 μg carbohydrate in 10 μl) was run in the first dimension against homologous antiserum. (d) EDTA-extracted OM complex from a clinical isolate (20 μg protein in 10 μl) was run against antiserum to the reference strain. The results demonstrate the presence of a heat-stable, aqueous phenol-extractable common antigen in the two strains. (Photograph kindly supplied by G. Cousland).

should be preserved, loss of immunogenicity is often inevitable.

Culture of bacteria

As the growth conditions of the organism can markedly affect the expression of antigens, factors such as medium composition, pH, incubation temperature, oxygen tension, phase of growth at harvesting, and the choice between batch or continuous culture need to be considered. A compromise must usually be made between a medium approximating the situation in the

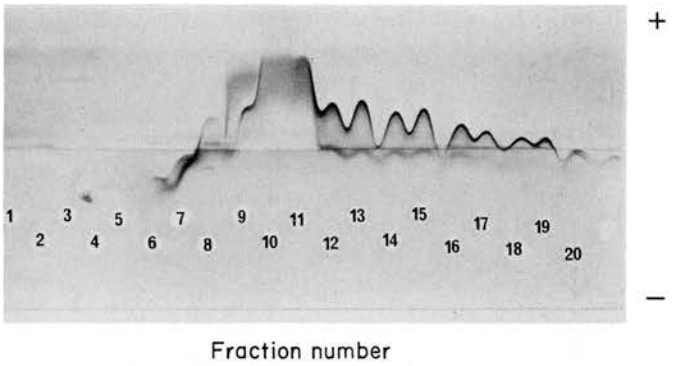

Fig. 4.3. Fused rocket immunoelectrophoresis of a 40% (w/v) aqueous phenol extract of cytoplasmic membranes from *Clostridium difficile* fractionated on an immunosorbent column (antiserum to whole cells of *C. difficile* linked to CNBr-activated Sepharose 4B) by elution with 50% (v/v) ethylene glycol in 5 mM-lysine, 140 mM-KCl (pH 11.5). Volumes (5 μl) of each fraction were placed in wells, allowed to diffuse for 1 h at 4°C and electrophoresed into antiserum to whole cells of *C. difficile* (200 μl serum in 5 ml agarose) for 16 h at 12 V cm^{-1}. Two distinct antigens were demonstrated.

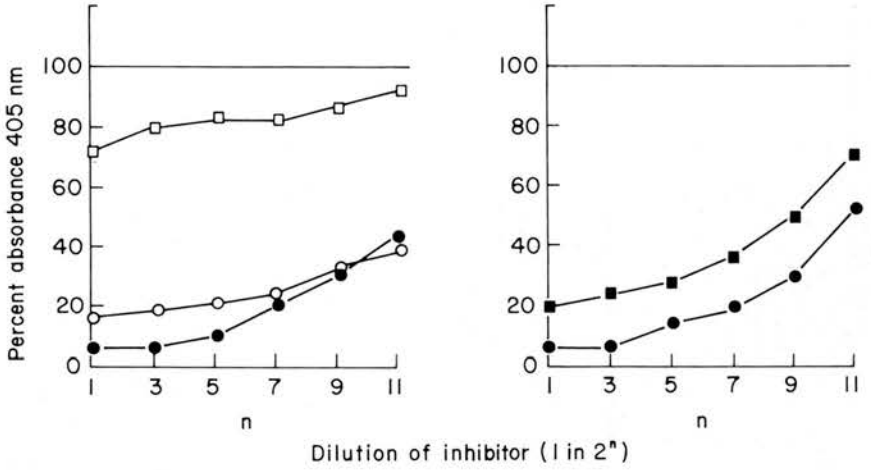

Fig. 4.4. Determination of the chemical nature of the major surface antigens of *Bacteroides fragilis* by inhibition of ELISA. An uninhibited indirect ELISA was performed to obtain the 100% value (——). *B. fragilis* outer membrane (OM) complex was coated on to wells of a polystyrene multi-well plate and reacted with doubling dilutions of antiserum raised in rabbits against whole cells of *B. fragilis*. The coated wells were reacted with anti-rabbit IgG-alkaline phosphatase conjugate followed by *p*-dinitrophenyl phosphate. The endpoint of the reaction was the highest dilution (titre) of antiserum that gave an absorbance reading of greater than 1.0 at 405 nm.

To determine the chemical nature of the major antigens detected above, an inhibition of ELISA was performed. *B. fragilis* OM complex was coated onto wells. Before the antiserum was added, doubling dilutions of potential inhibitors were mixed with an equal volume of antiserum diluted to twice the titre determined in the uninhibited assay and incubated at 37 °C for 30 min. Inhibitors were a positive control of untreated OM complex (●), OM complex heated at 121 °C for 15 min (○), OM complex treated with 0.01 M-$NaIO_4$ (pH 5) for 16 h at 20 °C (□), and lipopolysaccharide (■). After incubation with the inhibitors, the antiserum was added to the antigen coated wells and the assay completed as above.

The results showed that heat-stable, periodate-labile components of the OM complex are the major antigens detected by ELISA and that these are predominantly lipopolysaccharide [155].

host animal that may give high yields of an antigen but results in difficult purification. A simple synthetic medium provides a low yield but purification is easier.

Fractionation of the bacteria

When fractionating the bacterial cell the following points must be considered: (1) maintain efficient cooling during cell breakage as overheating may result in proteins being denatured; (2) minimize the action of autolytic enzymes, either by maintaining low temperature and rapid processing if heat-labile antigens are to be prepared or by inactivation by heat or formaldehyde if the antigens are heat stable; and (3) consider using chemical or enzymatic methods such as EDTA detergents or lysoyme for cell lysis in certain prepara-

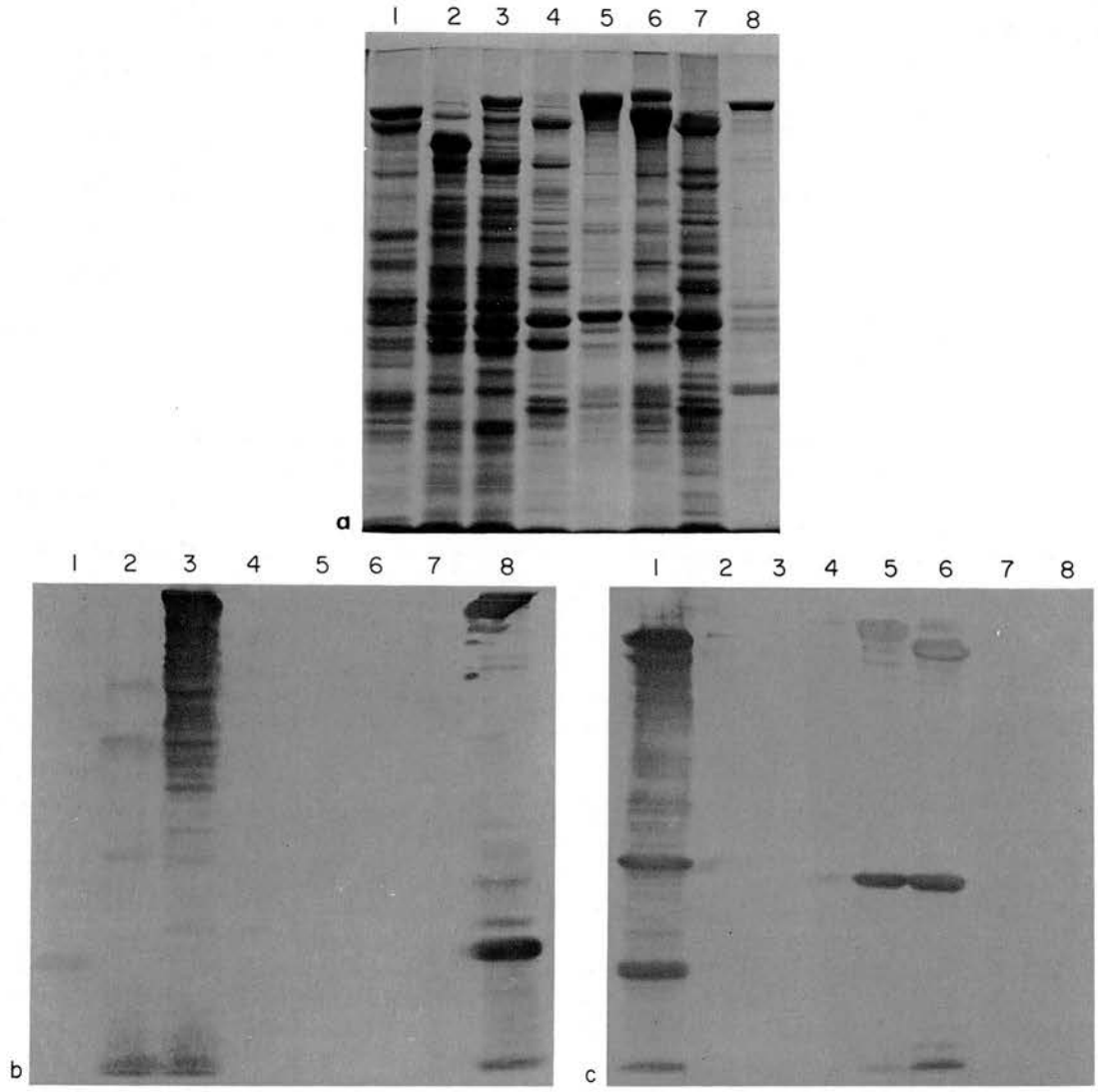

Fig. 4.5. SDS-polyacrylamide gel electrophoresis and corresponding electroblot transfers of EDTA-extracts of strains of *C. botulinum*, *C. novyi*, and *C. sporogenes*. Approximately 50 μg protein in 50 μl sample buffer were run on each track of a 10% acrylamide gel. Fig. 4.5a Coomassie blue stained gel: track 1, *C. novyi* type A NCTC 538; track 2, *C. botulinum* A1; track 3, *C. botulinum* pB25; track 4, *C. botulinum* npB188; track 5, *C. botulinum* C205; track 6, *C. botulinum* D154; track 7, *C. botulinum* E34; track 8, *C. sporogenes* NCTC 8594. Fig. 4.5b. Electroblot transfer of Fig 4.5a reacted with antiserum to *C. sporogenes* NCTC 8594. Fig. 4.5c. Electroblot transfer of Fig. 4.5a reacted with antiserum to *C. novyi* NCTC 538. SDS-PAGE was performed as in [136] and electroblot transfer as in [150]. Full details are as in [156].

tions—outer membrane preparations or ribosomal vaccines.

Purification

The newly developed high-resolution biochemical separation techniques should be used whenever possible. Immunoabsorbent affinity methods are especially useful. As many bacterial antigens of current interest are located at the cell surface, they are often firmly associated with membrane components. This necessitates the inclusion of detergents (Triton X-100 or sodium deoxycholate) in the purification schemes. These detergents often pose problems. They can

denature antigens and, more importantly, are often difficult to remove from the purified antigen. These points must be considered and those detergents that are recognized as causing extensive denaturation (sodium dodecyl sulphate) should be avoided whenever possible. Recently, Pierce and Warriner have introduced an affinity chromatography medium for removal of detergents from protein solutions.

Identification of antigens

Avoid the older immunological methods that have low resolving power. As pointed out above, detergents are often necessary for the purification of membrane antigens. These detergents are often included in identification systems to avoid reaggregation and complexing of antigens. For example, it is routine to include 1% Triton in crossed immunoelectrophoresis gels when membrane antigens are investigated.

Non-immunological analytical techniques should also be included to detect the presence of non-antigenic contaminants. When using either technique, it is worthwhile overloading the system with antigen to determine minor contaminants.

Sources of technical information

As well as the references listed in this chapter, the reader is encouraged to consult other sources for general descriptions of methods and techniques. The various 'Methods in ...' series (Academic Press, London and New York) are useful, i.e. 'Methods in Microbiology' and 'Methods in Enzymology'. The catalogues and pamphlets issued free of charge by many of the chemical manufacturers are invaluable sources of technical information; those produced by Pharmacia, BioRad, LKB, and Miles are especially worthy of note.

Appendix

Addresses of suppliers of equipment and materials

Albright and Wilson, Whitehaven, Cumbria, U.K.
Amicon Corporation, Lexington, MA, U.S.A.
Aminco, American Instrument Company Inc., Silver Springs, MD, U.S.A.
B.D.H. Chemicals, Ltd., Poole, Dorset BH12 4NN, U.K.
Bio-Rad, 32nd and Griffin Ave., Richmond, CA 94804, U.S.A.
Braun Melsungen Int. Gmbtt., Schwarzenberger Weg 23, D3508, Melsungen, W. Germany.
Dawe Instruments, Ltd., Concord Road, Western Ave., London W3 05D, U.K.
Difco Laboratories, Detroit, MI, U.S.A.
Dupont Company, Newton, CT. 06470, U.S.A.
L.K.B. Products AB, Box 305, S-16126 Bromma, Sweden.
Miles Laboratories Ltd., P.O. Box 37, Stoke Poges, Slough SL2 4LY, U.K.
Millipore Corporation, Bedford, MA 01730, U.S.A.
M.S.E. Scientific Instruments, Manor Royal, Crawley, W. Sussex, U.K.
Oxoid Ltd., Wade Road, Basingstoke, Hants. RQ24 0PW, U.K.
Pharmacia Fine Chemicals AB, Box 175, S-75104 Uppsala 1, Sweden.
Pierce and Warriner (U.K.) Ltd., 44 Upper Northgate Street, Chester, U.K.
Sigma Chemical Co., St. Louis, MO 63178, U.S.A.
Vortex Mixers, Hampton, Middlesex, U.K.
Waring Products Division, Route 44, New Hartford, CT, U.S.A.
Whatman W. and R. Balston (Modified Cellulose Ltd.), Springfield Mill, Maidstone, Kent, U.K.

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Immunoblotting techniques

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Introduction

Immunoblotting or western blotting is the technique by which macromolecules, usually proteins, are separated by polyacrylamide gel electrophoresis and are then electrophoretically transferred to a nitrocellulose or similar membrane, where they are probed with antibody to locate antigens. Since the transfer method was first developed about 10 years ago by Towbin *et al.* (*Proc Natl Acad Sci USA* 1979, 76:4350–4354), it has become one of the most widely used immunological techniques with an ever-increasing range of applications and modifications. It has largely superseded the traditional immunoelectrophoresis techniques such as crossed and rocket immunoelectrophoresis. It is used in all areas of immunological research, in diagnostic serology, and it is becoming the standard method throughout the biological sciences for the high-resolution detection of any molecule for which an antibody is available. In this review, I shall attempt to describe the current state of the technique and then go on to discuss some of the most recent applications; it is not possible to cover all of these here, so I shall concentrate on those in the fields of immunology and microbiology. For an extensive, recent 'state-of-the-art review', see [1].

The state of the technique

In immunoblotting, antigens are electrophoretically transferred, after separation, to nitrocellulose or to one of the more expensive, but much more robust, cationic nylon membranes, which have higher binding capacities for most protein antigens (Miribel and Arnaud, *J Immunol Methods* 1988, 107:253–259). The binding of the protein to the membrane is thought to be mediated largely by hydrophobic interactions. The transfer apparatus was originally a large tank of several litres capacity in which the gel and transfer membrane were mounted in a cassette between platinum wire electrodes. Large volumes of relatively expensive buffer were required for this apparatus. However, the more recently developed semi-dry transfer apparatus with two flat graphite electrodes is gaining popularity. There are savings in buffer costs and the transfer is more rapid because of the much steeper voltage gradient. An extension to the original concept in which the electrophoretic transfer step is circumvented is the application of the antigen directly to the membrane as a dot (dot blotting) [1]. A further modification to this technique is the recently described line immunobinding assay [2], in which several narrow lines of different antigens are sprayed horizontally across a sheet of nitrocellulose membrane. The sheet is then cut into vertical strips before probing with serum. This technique has been successfully applied to the serological investigation of several viral infections. An extremely similar, but more appealing, 'line blot' in which lines of antigen are applied with an ink pen point, has also been described [3].

The main problems encountered with immunoblotting include: (1) difficulty with the transfer or lack of binding of the antigen; (2) the antigen losing its antibody-binding capacity after separation and transfer, presumably by denaturation, and the subsequent need for renaturation of the antigen; (3) high background staining or non-specific binding, and the need for blocking agents; (4) lower sensitivity than enzyme-linked immunosorbent assays (ELISAs); (5) quantification of the results; and (6) the difficulty of miniaturising the technique for use in large-scale screening. Many of these problems have been addressed recently, with a limited amount of success [1]. Of these problems, the two areas that are still subject to the most debate and confusion are the denaturation and renaturation of antigens, and the role of blocking agents and detergents.

In the standard transfer method in a large tank, any ionic detergent, such as sodium dodecyl sulphate (SDS), used in the polyacrylamide gel electrophoresis is removed or at least greatly diluted. Otherwise, the SDS would inhibit the binding of the antigen to the membrane and, subsequently, of the antibody to the antigen. In the semi-dry system, therefore, the gel must first be washed. The step after transfer is usually to block any unbound sites on the membrane with a protein such as gelatin or bovine serum albumin to prevent non-specific antibody binding. All subsequent buffers usually contain the non-ionic detergent Tween 20 to prevent non-specific binding. Re-

Abbreviations

CN—4-chloro-1-naphthol; **DAB**—3, 3′ diaminobenzidine tetrahydrochloride; **ELISAs**—enzyme-linked immunosorbent assays; **HIV**—human immunodeficiency virus; **LPS**—lipopolysaccharide; **RNP**—ribonucleoprotein; **SDS**—sodium dodecyl sulphate; **SLE**—systemic lupus erythematosus.

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cently, it has been suggested that a blocking agent and a blocking step are unnecessary if Tween 20 is simply included in all steps after transfer [4]. Contrary to this is the report [5] that Tween 20 mediates a non-specific binding of enzyme-labelled or radio-labelled IgG to allergenic extracts of oak pollen. The authors admit that this may be specific to pollen proteins but suggest caution. Others have suggested that the use of Tween 20 can result in the loss of protein from nitrocellulose (Hoffman and Jump, *J Immunol Methods* 1986, 194:191–196).

Many workers are still adamant that blocking is essential, especially when working at the limits of the technique, where high concentrations of serum and long incubation times are needed to detect low levels of antibody. In addition, blocking is often necessary where background staining is a problem, especially with some of the non-nitrocellulose membranes. If blocking is considered necessary, then it is important not to overdo it. As the antigens bind through hydrophobic interactions, it is thought that the blocking protein slowly exchanges with the bound antigen, resulting in a loss of antigen. The extent of the loss is directly proportional to the concentration of blocking protein and to the length of time of exposure to it. In a recent quantitative investigation [6], using powdered low-fat milk, an extremely inexpensive and very popular blocking agent, it was shown that as much as 25% of the antigen could be lost in a 24 h period. The authors recommend the use of 0.5% milk for 30 min with 0.2 μm pore-size nitrocellulose as a suitable blocking procedure.

Turning to the problem of denaturation, it is perhaps not surprising that some protein antigens lose their ability to bind antibody after heating at 100°C in an SDS-solubilizing buffer, followed by separation and transfer. This is especially apparent when blotting with monoclonal antibodies. It is thought, however, that a considerable degree of renaturation occurs during the transfer and subsequent washing and blocking. Some outer membrane proteins of the meningococcus require incubation with zwitterionic detergents or ionic detergents containing substituted quaternary ammonium or amino groups, or with specific meningococcal lipopolysaccharide (LPS) to restore antibody binding. Non-ionic detergents such as Triton X100 or Tween 20 do not work well [7].

In summary, it seems sensible that for any new system of antigen/antibody binding the simplest technique is tried first, *viz.* using no blocking agent but only Tween 20 throughout, subsequent to transfer. If problems are encountered with high background, then the use of defatted powdered milk as a blocking agent should be considered. If problems are still experienced, other blocking agents or ways of making the assay more sensitive should be tried.

As with all immunoassays, many amplification methods have been used to increase the sensitivity of immunoblotting. Most of these methods involve streptavidin–biotin binding [1]. Recently, two other approaches have been investigated. The first [8] is extremely simple and involves using a mixed chromogenic substrate for the horseradish peroxidase conjugate. Mixing the two commonly used substrates, 3, 3′ diaminobenzidine tetrahydro-chloride (DAB) and 4-chloro-1-naphthol (CN), results in a 10-fold increase in sensitivity and the product is much easier to record by photography. Ikegaki and Kennet [9] claim that their method also allows a significant increase in sensitivity. After incubation of the first antibody, the antigen antibody complex on the nitrocellulose is treated with glutaraldehyde. A several-fold increase in sensitivity was observed with concentrations of glutaraldehyde in the range 0.1–0.25%; this was accompanied by a decrease in non-specific binding of secondary or tertiary reagents. Concentrations of 0.5% glutaraldehyde and above resulted in loss of sensitivity.

Non-electrophoretic affinity immunoblotting is a recently developed technique (Knisley and Rodkey, *J Immunol Methods* 1986, 95:79–87). A sheet of nitrocellulose is soaked in specific antigen and, after drying and blocking, is placed in direct contact with isoelectrofocused immunoglobulins to allow the binding of specific antibodies by capillary attraction. This method has been used mainly for the analysis of clonotype distribution. An example of its application in the investigation of congenital human immunodeficiency virus (HIV) infection is described below.

Applications in immunology

The areas of immunology in which immunoblotting is advancing our knowledge to the greatest extent are autoimmune disease and allergy. Both of these areas involve the identification of specific antigens among complex mixtures, something that has, until recently, been difficult to achieve.

Autoimmune disease

The detection and quantification of autoantibodies in the diagnosis and pathogenesis of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and primary immune neutropenia are obviously important. Only with the advent of immunoblotting have investigations such as those described below been possible.

Nuclear antigens are important markers in rheumatoid arthritis, SLE and connective tissue disease in general. Well known antigens such as the Sm and ribonucleoprotein (RNP) nuclear antigens have been investigated by immunoblotting [10]. Antibodies to Sm are highly specific to SLE, whereas anti-RNP antibodies are associated with mixed connective tissue disease. Using immunoblotting, De Keyser *et al.* [11] have detected a new anti-nuclear antibody in patients with rheumatoid arthritis.

Immunoblotting has been used in an interesting way in the investigation of T-cell activation in SLE [12]. Nuclear protein antigens were first located by immunoblotting. The areas of nitrocellulose containing the antigens were then dissolved in dimethyl sulphoxide and nitro-

cellulose/antigen particles were precipitated with carbonate bicarbonate buffer. The particles were then used in T-cell-enriched lymphocyte proliferation assays. Immunoblotting has also allowed the identification of neutrophil antibodies in autoimmune neutropenia [13].

Allergy

The identification of allergens has been greatly facilitated by immunoblotting (Tovey and Baldo, *Electrophoresis* 1987, 8:452). IgE, as well as other immunoglobulins directed against specific allergens, can be easily detected and quantified by immunoblotting. Two recent examples demonstrate the technique's value. In the first, an investigation of farmers' lung (extrinsic allergic alveolitis) caused by *Micropolyspora faeni* showed that patients characteristically have high antibody levels to three specific fungal proteins, which may be useful in diagnosis [14]. In the second example, IgE and IgG in patients suffering from Type 1 allergy to birch pollen were investigated by immunoblotting [15]. Of the 58 patients, 56 had IgE antibodies that were reactive with a 17 kD Bet v I antigen. In 33 of these 56 patients, it was the only antigen to which IgE was directed. All the pollen proteins were recognized by IgG antibodies, but only weakly in the case of the 17 kD antigen. The authors suggest that low or absent IgG may facilitate the allergic reaction.

Other applications

Two other papers are worthy of note in the general field of immunology. One describes the analysis of complement Factor B activation by immunoblotting [16], allowing analysis in complex biological fluids without purification. The other highlights the use of the immunoblotting technique in clinical chemistry [17], where a method has been developed for a subclass typing of human IgG myeloma paraproteins.

Applications in microbiology

There is a vast literature on immunoblotting applications in microbiology. The areas on which I shall concentrate are the serodiagnosis of infectious disease, particularly HIV diagnosis, and the use of immunoblot fingerprinting in epidemiological studies of bacterial and fungal infections.

Immunoblot analysis is a valuable confirmatory test for the detection of antibodies to HIV. Its use is recommended for sera giving positive or borderline results in enzyme immunoasays. Nitrocellulose strips of separated HIV antigens permit simultaneous detection of antibodies to distinct HIV antigens. Those antigens considered important include the transmembrane glycoprotein (gp41) and the core proteins p55 and p24. Several licensed commercial test strips are now available that avoid some of the earlier problems of non-uniform antigen preparations. Despite this, there are frequent false-positive or 'intermediate' or 'indeterminate' immunoblot results (Meyer and Pauker, *N Engl J Med* 1987, 317:238–241). Because of the ethical, medico–legal and emotive consequences of false positives, especially in blood donor screening, a great effort is being made to clarify the immunoblot assay [18–20]. Aw (this issue, pp 892–897) discusses HIV surveillance in more detail.

In infants delivered to HIV-positive mothers it is important to be able to detect congenital infections. Using conventional serological techniques, infections cannot be detected in infants of less than 15 months of age because of the difficulty in discriminating between passively acquired maternal antibody and actively produced antibody, but affinity immunoblotting has been developed to detect infants' antibody as early as 2 months [21].

Since immunoblotting was first used to fingerprint *Clostridium difficile* isolates involved in an outbreak of antibiotic-associated pseudomembranous colitis (Poxton *et al.*, *J Med Microbiol* 1984, 17:317–324), its use has been extended to many hospital pathogens, both bacterial and fungal, which have been difficult to type by other means (Burnie and Matthews, *J Immunol Methods* 1987, 100:41–46). The technique has proved especially useful for methicillin-resistant *Staphylococcus aureus* [22], where exported proteins (culture supernatants) provide a good source of antigen. A recent example of the use of the technique in a fungal infection is described by Burnie *et al.* [23].

Conclusions

Immunoblotting is an extremely powerful technique which in the past 10 years has revolutionized the detection of both antigens and antibodies. The high resolving power of this immunoassay permits applications to extremely complex mixtures of antigens which were hitherto impossible. Like many such separation techniques, it is almost more of an art than a science and some of the mysteries of the binding mechanisms involved in immunoblotting and the use of detergents and blocking agents have still to be explained. It still has some way to go to reach its full potential.

Annotated references and recommended reading

- • Of interest
- •• Of outstanding interest

1. •• STOTT DI: Immunoblotting and dot blotting. *J Immunol Methods* 1989, 119:153–187.

A comprehensive critical review. Includes methodology, design of equipment, types of gel, membrane transfer buffers, blocking agents, probes, a discussion of denaturation of proteins, detection methods, quantification and special applications.

2. • AEPPLI RE, BARGETZI MJ, BINZ H: Diagnostic screening of multiple antigen-antibody reactions in a new single assay on nitrocellulose: the line immunobinding assay (LIBA). *J Immunol Methods* 1989, 120:93–98.

Using a fine spray, several different antigens are applied as lines horizontally across nitrocellulose. Vertical strips are probed with serum for the detection of antibodies. This technique has been applied to the serology of rubella, measles and mumps. Compares favourably with ELISA.

3. • RAOULT D, DASCH GA: **The line blot: an immunoassay for monoclonal and other antibodies. Its application to the serotyping of Gram-negative bacteria.** *J Immunol Methods* 1989, 125:57–65.

Extremely similar to the technique described in [2] but much simpler to use. A simple ink pen and a ruler are used to draw lines of antigen across a sheet of nitrocellulose. Uses less than 0.25 μg antigen per assay. It has been applied to serotyping the following Gram-negative genera: *Proteus, Rickettsia, Rochalimaea* and *Legionella*.

4. • MOHAMMAD K, ESEN A: **A blocking agent and a blocking step are not needed in ELISA, immunostaining dot-blots and western blots.** *J Immunol Methods* 1989, 117:141–145.

If Tween 20 at 0.05% is included in all steps subsequent to transfer, significant savings in time and money are made, without loss of sensitivity.

5. • LORIA RC, PRANGE JO, WEDNER HJ: **Immunoblotting of pollen proteins: Tween 20 mediates nonspecific binding of gammaglobulins to immunoblots of oak-pollen proteins.** *J Allergy Clin Immunol* 1988, 82:834–841.

Claimed to be the first report of non-specific binding in immunoblotting of an allergenic extract, this paper confirms that Tween 20 results in non-specific binding. It binds to pollen grains and allows binding of labelled probes—both horseradish peroxidase and ^{125}I-labelled anti-human IgE. This also occurs with Triton X100. Bovine serum albumin and gelatin led to a marked reduction in non-specific binding. Although admitting that this phenomenon may be restricted to pollen proteins, the authors rightly suggest caution.

6. • DENHOLLANDER N, BEFUS D: **The loss of antigens from immunoblotting membranes.** *J Immunol Methods* 1989, 122:129–135.

Blocking with low-fat milk powder can result in the non-selective loss of antigen from membranes. The loss is probably due to the simple exchange of one protein for another.

7. • WEDEGE E, BRYN K, FROHOLM LO: **Restoration of antibody binding to blotted meningococcal outer membrane proteins using various detergents.** *J Immunol Methods* 1988, 113:51–59.

Some heat-denatured meningococcal outer membrane proteins do not bind antibody if immunoblotted. Binding can be restored if zwitterionic and ionic detergents with substituted quaternary ammonium or amino groups with a minimum of 10 carbon atoms in the alkyl chain are used. Meningococcal LPS can also restore binding to a class 2 porin (outer membrane) protein, but other LPSs cannot.

8. • YOUNG PR: **Enhancement of immunoblot staining using a mixed chromogenic substrate.** *J Immunol Methods* 1989, 121:295–296.

One horseradish peroxidase substrate, DAB, is a sensitive substrate but the coloured product is pale and photographs poorly. The other commonly used substrate, CN, although giving a better contrasting purple colour, is slightly less sensitive. A combination of the two results in a synergistic increase in sensitivity and a high-contrast black colour that is suitable for photography. Just before use, the substrates are mixed with phosphate-buffered saline and hydrogen peroxide.

9. • IKEGAKI N, KENNET RH: **Glutaraldehyde fixation of the primary antibody-antigen complex on nitrocellulose paper increases the overall sensitivity of immunoblot assay.** *J Immunol Methods* 1989, 124:205–210.

A simple additional step to any standard immunoblot assay. The authors recommend a 15 min incubation with 0.25% glutaraldehyde in phosphate-buffered saline immediately after incubation with the first antibody.

10. • COMBE B, RUCHETON M, GRAAFLAND H, LUSSIEZ V, BRUNEL C, SANY J: **Clinical significance of anti-RNP and anti-Sm autoantibodies as determined by immunoblotting and immunoprecipitation in sera from patients with connective tissue diseases.** *Clin Exp Immunol* 1989, 75:18–24.

Immunoblotting can be used as a highly sensitive method for detection of anti-Sm and anti-RNP antibodies. Anti-Sm antibodies are highly specific for SLE, being found in 76% of SLE patients. Anti-RNP antibodies are associated with mixed connective tissue disease, which may be considered a distinct clinical entity associated with a specific serological marker.

11. • DE KEYSER F, VERBRUGGEN G, VEYS EM: **Identification of a new antinuclear antibody by immunoblotting.** *Lancet* 1989, ii:927.

Use of immunoblotting to screen for anti-nuclear antibodies in patients with rheumatic diseases. Antibodies that are not detectable by immunoprecipitation in gels are identified by immunoblotting.

12. • PHAM BN, PRIN D, GOSSET D, HATRON PY, DEVULDER B, CAPRON A, DESSAINT JP: **T-lymphocyte activation in systemic lupus erythematosus analysed by proliferative response to nucleoplasmic proteins on nitrocellulose immunoblots.** *Clin Exp Immunol* 1989, 77:168–174.

Suggests that activated T cells contribute to fine modulation of B-cell reactivity in SLE. Antigens are solubilized as nitrocellulose particles.

13. • ROTHKO K, KICKLER TS, CLAY MC, JOHNSON RJ, STRONCEK DF: **Immunoblotting characterization of neutrophil antigenic targets in autoimmune neutropenia.** *Blood* 1989, 74:1698–1703.

Demonstration that immunoblotting may be used to identify antigenic targets in autoimmune neutropenia that are not definable by other serological tests.

14. • IRANITALAB M, JAROLIM E, RUMPOLD H, STEINER R, EBNER H, FELDNER H, SCHEINER O, KRAFT D: **Characterization of *Micropolyspora faeni* antigens by human antibodies and immunoblot analysis.** *Allergy* 1989, 44:314–321.

Identification of fungal antigens from patients with farmers' lung by measuring IgG, IgM and IgA antibodies. Stresses the need for *M. faeni* antigens of 11, 25, and 60 kD for *in vitro* diagnosis.

15. • JAROLIM E, RUMPOLD H, ENDLER AT, EBNER H, BREITENBACH M, SCHEINER O, KRAFT D: **IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of *Betula verrucosa*.** *Allergy* 1989, 44:385–395.

Results of two-dimensional electrophoresis/immunoblot demonstrates heterogeneity in birch pollens. Suggestion that IgG directed against minor allergens may function as trapping antibody in healthy individuals.

16. • DORAN M, REEN DJ: **Analysis of complement Factor B activation by PAGE immunoblotting.** *J Immunol Methods* 1989, 118:237–243.

A sensitive and quantitative tool for the analysis of Factor B in complex biological fluids. Uses rabbit anti-human complement Factor B antiserum.

17. • FASULLO FJ, FRITCHE HA, LIU FJ, HAMILTON RG: **IgG heavy-chain subclass typing of myeloma paraproteins by isoelectric focusing immunoblot analysis.** *Clin Chem* 1989, 35:364–368.

Use of IgG_{1-4} monoclonal antibodies. Allows the monitoring of pI and subclass of an IgG paraprotein over the course of a patient's therapy programme.

18. • GENESCA J, JETT BW, EPSTEIN JS, SHIH JW-K, HEWLETT IK, ALTER HJ: **What do western blot indeterminate patterns for human immunodeficiency virus mean in EIA-negative blood donors?** *Lancet* 1989, ii:1023–1025.

It is prudent to continue to exclude blood units that are enzyme-immunoassay positive and indeterminate, and to follow-up donors. However, indeterminate donors who have no history of exposure to HIV-1 and whose indeterminate pattern does not become positive in 6 months are unlikely to have HIV infection.

19. • ZOLLA-PAZNER S, GORNAY MK, HONNEN WJ, PINTER A: **Reinterpretation of human immunodeficiency virus western blot patterns.** *N Engl J Med* 1989, 320:1280–1281.

Viral antigens on commercial nitrocellulose strips with apparent molecular masses in the 120–160 kD range are not the gp120 envelope gly-

coprotein or the gp160 envelope precursor, but are primary multimers of the gp41 transmembrane glycoprotein.

20. • LANTIN JP, GRAF I, FREI PC: **Serological diagnosis of HIV infection: incidence, outcome and significance of partial reactions in western blot analysis.** *Clin Exp Immunol* 1989, 76:332–336.

Investigates intermediate western blot patterns (1–3 'specific' bands). Similar findings to [18]. Stresses the importance of further testing 3 months later.

21. •• SLADE HB, PICA RV, PAHWA SG: **Detection of HIV-specific antibodies in infancy by isoelectric focusing and affinity immunoblotting.** *J Infect Dis* 1989, 160:126–130.

Nitrocellulose was coated with synthetic gp41 antigen and used to affinity-blot serum separated by isoelectric focusing. The presence or absence of clonotypically distinct bands of IgG correlated with infection status.

22. • THOMSON CARTER FM, PENNINGTON TH: **Characterisation of methicillin-resistant isolates of *Staphylococcus aureus* by analysis of whole-cell and exported proteins.** *J Med Microbiol* 1989, 28:25–32.

Immunoblots of culture supernatants were preferable to whole-cell proteins in discrimination between stains. Pooled human plasma known to contain antibodies to many *S. aureus* polypeptides was used as the primary probe.

23. • BURNIE JP, MATTHEWS RC, CLARK I, MILNE LJR: **Immunoblot finger-printing of *Aspergillus fumigatus*.** *J Immunol Methods* 1989, 118:179–186.

First immunologically based typing system for *Aspergillus fumigatus*. Uses rabbit antiserum to detect up to 16 antigenic bands and gives better discrimination than silver staining.

Bacterial Cell Surface Techniques

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Contents

1.13 Determinants of bacterial virulence

I. R. Poxton and J. P. Arbuthnott

Bacterial virulence determinants can be defined as the components and products of the bacterial cell which confer on the bacterium the potential to harm the host. In very few instances is it possible to define a single such factor. This is perhaps possible only in certain of the classic bacterial intoxications (e.g. botulism) where the powerful exotoxin may be considered to be the sole determinant of virulence. In most cases the damage suffered by the host is multifactorial, the factors involved including those of host origin as well as bacterial factors. For example, in certain *Escherichia coli*-induced diarrhoeas in piglets, the organism requires two virulence determinants: the surface fimbrial antigen to allow adherence to the mucosa, and the ability to secrete one or more enterotoxins. The animal host must be of a certain genotype to express the receptors for the adherence factor and its age is often important. Examples of these, together with many others illustrating the complexity of bacterial virulence, are described in more detail later in this chapter.

The virulence determinants of bacteria are conveniently divided into those that are **cell associated** (i.e. cell surface components) and those that are **extracellular** (i.e. the classic exotoxins and extracellular enzymes). It should be stressed that many of the virulence factors described here have functions other than those of damaging the host. Most have a role, which is often primary, in the anatomy or physiology of the bacterial cell, and that of a virulence factor is purely coincidental.

Useful reviews on virulence and pathogenesis include those of Roth (1988) and Brubaker (1985); for a more historical perspective, see Howie and O'Hea (1955) and Arbuthnott *et al.* (1983).

Surface virulence determinants

Before discussing the various surface virulence determinants, it is appropriate to describe the stages of the pathogenic processes, particularly those at which surface factors may be important. For a detailed review of pathogenesis, the reader is referred to the monograph by Mims (1987). Reviews more specifically relating to surface virulence factors include: Smith (1977), Easmon *et al.* (1983) and Urbaschek (1987).

Surface factors and pathogenesis

Once a pathogenic bacterium is in or on a susceptible host, it must become established at a suitable site. For example, at mucous membranes it is usual for the pathogen to penetrate the mucous blanket and subsequently to adhere to the epithelium. Movement through the mucus to the epithelial cells may be dependent on chemoattractants in host tissue, and the bacterium may require flagella and possibly mucus-degrading enzymes. Adherence to the tissue is then usual to avoid the mechanical action of the cilia or the flushing action of the body fluids. Surface factors involved in adherence include **fimbriae (pili), lipoteichoic acids, exopolysaccharides** (capsules and slime) and **outer membrane proteins**. These factors may act in conjunction with host-derived polymers and those from other bacteria to produce a glycocalyx or biofilm, e.g. in dental plaque (see Chapter 12). The binding of the receptor on the host cell surface and the bacterial adhesin is often a highly specific 'lock and key' association. The host cell surface protein, fibronectin, is a receptor for several pathogens and is discussed later.

Once the organism is located in the host, subsequent factors in the pathogenic process include evasion of the host defence system and the ability of the pathogen to compete with the commensal flora for nutrients and space. Bacterial factors for the former step include possession of a capsule, which can be both antiphagocytic and a camouflaging mechanism if it mimics host tissue. The long polysaccharide chains of 'smooth' lipopolysaccharide as well as of capsular polysaccharide allow the fixation of complement at a site distant from the bacterial cell membrane, thus making the bacterium resistant to the lethal lytic effect of normal serum. Some bacteria are classically intracellular and thus evade the defence mechanisms by their internal location. Factors which confer virulence on pathogens by allowing them to compete with the normal flora include the ability to secrete **antibacterial substances** (e.g. bacteriocins, organic acids, alcohols) and their ability to express **scavenging mechanisms** such as iron-binding proteins.

Subsequent steps in pathogenesis involve the mechanisms by which the host is harmed. It is at this stage that **toxins** – both the classic exotoxins and, in the case of gram-negative bacteria, the lipopolysaccharide (endotoxin) – exert their main influence. Their effects can be either local or systemic. The actions of the toxins are described in detail later. Tissue damage can also result from direct invasion of tissue, from inflammation or from harmful immune responses, which include anaphylaxis, complement-mediated cytolysis, immune-complex reactions and cell-mediated reactions (see Chapters 14–17).

Finally, to complete the cyclic process of pathogenesis, the organism is released from the host and is disseminated to other susceptible hosts. The ability to survive outside the host could be taken as contributing to virulence. The waxy coat of the mycobacteria, the ability to form spores and the possession of a highly hydrated capsule are obvious examples of survival mechanisms but are not considered further in this chapter.

From this brief description of pathogenesis it is apparent that virulence factors are involved at all stages of the disease process and often the same component can be involved in more than one stage. Furthermore, the virulence determinants encompass the whole range of surface components. Table 13.1 summarizes the role of surface components as determinants of virulence.

The bacterial cell surface components will be described in the first part of this chapter. A brief description of their structures is followed by a discussion of their roles as virulence determinants, with special reference to the relationship, at the molecular level, between chemical and physical structure and virulence mechanisms.

Table 13.1 Bacterial cell surface components as virulence determinants

Surface component	Contribution to virulence
Capsule/exopolysaccharide	Adhesion, antiphagocytic camouflage from immune system, mimicry of host tissue, resistance to complement, invasiveness
Lipopolysaccharide	Resistance to complement, invasiveness, endotoxic stimulation of many harmful immune responses, adhesion
Teichoic acid/lipoteichoic acid	Adhesion, sequestration of divalent ions
Fimbriae/pili/fibrillae/colonization factors	Adhesion, antiphagocytic
Flagella/axial filaments	Chemotaxis, penetration of mucus, adhesion, intracellular survival
Outer membrane proteins	Adhesins, sequestration of iron, invasiveness, intracellular survival, resistance to complement
Surface proteins of gram-positive bacteria	Adhesion, binding to Fc region of immunoglobulins

Carbohydrate virulence factors: exopolysaccharides

Structure Exopolysaccharides include the discrete capsules of many pathogenic bacteria and the loosely associated slime produced by mucoid bacteria. They are the classic K antigens of the enterobacteria. Capsules were among the first known bacterial virulence determinants. The first demonstration of bacterial transformation by Griffiths (1928) involved the transformation of rough (non-capsulate, non-virulent) pneumococci to the smooth (capsulate, virulent) form.

Exopolysaccharides form highly hydrated, water-insoluble gels. They can be readily demonstrated in the light microscope by negative staining with indian ink. In the electron microscope a ruthenium red stain, which binds to negatively charged polymers, is usually used to reveal capsules (Hancock and Poxton 1988). The chemical composition of exopolysaccharides can be of two types: homo- and heteropolysaccharides, consisting respectively of a single sugar monomer, (e.g. the levans and dextrans of many oral streptococci) or of repeating oligosaccharide units of more than one monomer. Many exopolysaccharides are acidic due to the possession of carboxyl groups, either from acidic sugars such as uronic acids or neuraminic acid, or from non-sugar substituents such as pyruvyl, acetyl and formyl groups. Their acidic nature is reflected in many of their properties. A few of the bacilli (e.g. *Bacillus anthracis*) have a capsule made up of a single amino acid, poly-D-glutamic acid. In physical chemistry and function, however, it is similar to the polysaccharide capsules. Examples of the basic structures of the exopolysaccharides of several pathogenic bacteria are shown in Table 13.2, and the appearance of a capsulate bacterium is shown in Fig. 13.1. For more details of the structures of exopolysaccharides, see Sutherland (1977, 1985).

A relatively recent observation is that the long polysaccharide chains of some *E. coli* capsules terminate at their reducing end in a phosphodiester-linked lipid residue, which is often phosphatidic acid. It also seems likely that the terminal reducing sugar is 2-keto-3-deoxymannosoctonic acid (KDO: 3-deoxy-D-*manno*-2-octulosonic acid) whether or not this sugar is part of the structure of the oligosaccharide repeating unit. This question is reviewed by Jann and Jann (1983). It is probable that the lipid is involved in a hydrophobic interaction with the outer membrane, or other surface component, and anchors the capsule to the cell.

Biological activities As virulence factors, exopolysaccharides have two major roles. The first is in **adhesion** and is particularly obvious in *Streptococcus mutans*, the cariogenic organism. The bacterium can synthesize, from dietary sucrose, a branched, water-insoluble homopolymer of glucose which is dextran-like. It forms a glutinous layer on the surface of the tooth and contributes to the matrix of dental plaque. It also forms a metabolic substrate for acid formation. Recent evidence shows that coagulase-negative staphylococci adhere to prosthetic devices and catheters by means of an exopolysaccharide slime (Christensen *et al.* 1982, 1987).

The second main role of capsular polysaccharide in virulence is **protection of the organism** from the host defence systems. Since the classic work on the pneumococcus it has been recognized that capsules prevent bacteria from being engulfed by phagocytes. There are several possible reasons. Capsulate or slime-producing bacteria usually grow in microcolonies *in vivo* and engulfment is difficult on purely steric considerations. Furthermore, complement (see below) and opsonic antibodies cannot get access to the cell envelope. Another general observation is that the more hydrophilic the surface of a bacterium – and this can be related to the amount of polysaccharide – the less readily it can be phagocytosed. A good example of a potential virulence determinant with some of the above properties is the alginate slime of mucoid *Pseudomonas aeruginosa* in the lungs of children with cystic fibrosis.

Several species of bacteria produce a capsule with a chemical structure that mimics host tissue. This camouflages the organism from the immune system by appearing to the host as 'self' (Jann and Jann 1987). Perhaps the best known examples are the K1 capsule of *E. coli* and the immunologically and structurally identical capsule of *Neisseria meningitidis* group B. These organisms have capsules of α-2,8-linked *N*-acetylneuraminic acid which is partially O-acetylated. It cross-reacts immunologically with neonatal neural cell adhesion molecules (Hoffman *et al.* 1982). Capsules of similar composition are found in the important sheep pathogen *Pasteurella haemolytica* type A2 (Adlam *et al.* 1987). Another example of a capsule mimicking host tissue is provided by the hyaluronic acid capsules of *Str. pyogenes* and of *Past. multocida* type A. Many of these capsules also confer

Table 13.2 Examples of repeating unit structures for some capsular polysaccharides

Species	Group/type	Structure
Streptococcus pneumoniae	Type 1[a]	---GalUA $\xrightarrow[1,3]{}$ GlcNAc $\xrightarrow[1,3]{}$ GalUA---
	Type 6	$\xrightarrow[2]{}$ Gal $\xrightarrow[1,3]{\alpha}$ Glc $\xrightarrow[1,3]{\alpha}$ Rha $\xrightarrow[1,3]{\alpha}$ Rib–P $\xrightarrow[1]{}$
	Type 8	$\xrightarrow[4]{}$ Glc $\xrightarrow[1,3]{\beta}$ Glc $\xrightarrow[1,4]{\alpha}$ Gal $\xrightarrow[1,4]{\alpha}$ GlcUA $\xrightarrow[1]{\beta}$
Neisseria meningitidis	Group A[b]	$\xrightarrow[6]{}$ ManNAc-1-P ($\xrightarrow[1]{\alpha}$; 3-O-Ac)
	Group B	$\xrightarrow[8]{}$ NeuNAc $\xrightarrow[2]{\alpha}$
	Group C[b]	$\xrightarrow[9]{}$ NeuNAc ($\xrightarrow[2]{\alpha}$; 7/8-O-Ac)
Haemophilus influenzae	Type b[c]	$\xrightarrow[3]{}$ Rib $\xrightarrow[1,1]{\beta}$ Rib–P $\xrightarrow[5]{}$
Escherichia coli	K1[b,d]	$\xrightarrow[8]{}$ NeuNAc ($\xrightarrow[2]{\alpha}$; O-Ac)
Salmonella Typhi	Vi[b]	$\xrightarrow[4]{}$ GalNAcUA ($\xrightarrow[1]{\alpha}$; 3-O-Ac)

Data compiled from Jann and Westphal (1975), Lindberg (1977), Sutherland (1977), Robbins (1978) and De Voe (1982)

Abbreviations: Ac, acetyl; Gal, galactose; GalNAcUA, *N*-acetylgalactosaminuronic acid; GalU, galacturonic acid; Glc, glucose; GlcNAc, *N*-acetylglucosamine; ManNAc-1-P, *N*-acetylmannosamine phosphate; NeuNAc, *N*-acylneuraminic acid; Rha, rhamnose; Rib, ribose; Rib-P, ribitol phosphate

a Partial structure only

b Not all the sugar residues are O-acetylated

c An example of a teichoic acid polymer in a gram-negative organism

d Internal ester bridges are also present

resistance to the bactericidal effect of serum, and this is discussed further below.

As mentioned earlier, the capsule allows the bacterium to evade the immune system by making it resistant to the lytic action of complement. It is well known that, for lipopolysaccharide (LPS), which is described later, the longer the chains of O polysaccharide, the more resistant they are to complement. This is thought to be because the complement cascade is activated at a distance from the bacterial membrane so that no lysis occurs. It has been recognized for many years that the capsular polysaccharide also confers this property of serum resistance (Glynn and Howard 1970), especially the K1 antigen of *E. coli*. There was, however, a certain amount of confusion as to which component was most important in bacteria possessing both LPS and capsule. This problem has been largely clarified by the work of Cross and coworkers (1986), who concluded that serum resistance is multifactorial. The LPS of some *E. coli* serotypes is incapable of protecting against serum lysis. For these strains the capsule, which is most commonly K1, confers protection. For other serotypes of *E. coli* (e.g. 06) the capsule is much less important. This serotype often possesses the K5 capsule, which is as non-immunogenic as K1, but does not confer resistance to serum on the bacterium. Finally, it should be noted that the anticomplementary and antiphagocytic effects of capsules of *E. coli* and many other species are usually overcome by opsonization of the bacterium with capsule-specific antibodies.

There is a possible connection between the ability of the organism to resist the action of complement and its ability to invade the host; again, in *E. coli* the K1 antigen is important. Most bacterial pathogens found outside the gut and inside functional barriers, e.g. those causing bacteraemia, meningitis and urinary tract infections – especially pyelonephritis – are capsulate. One of the most

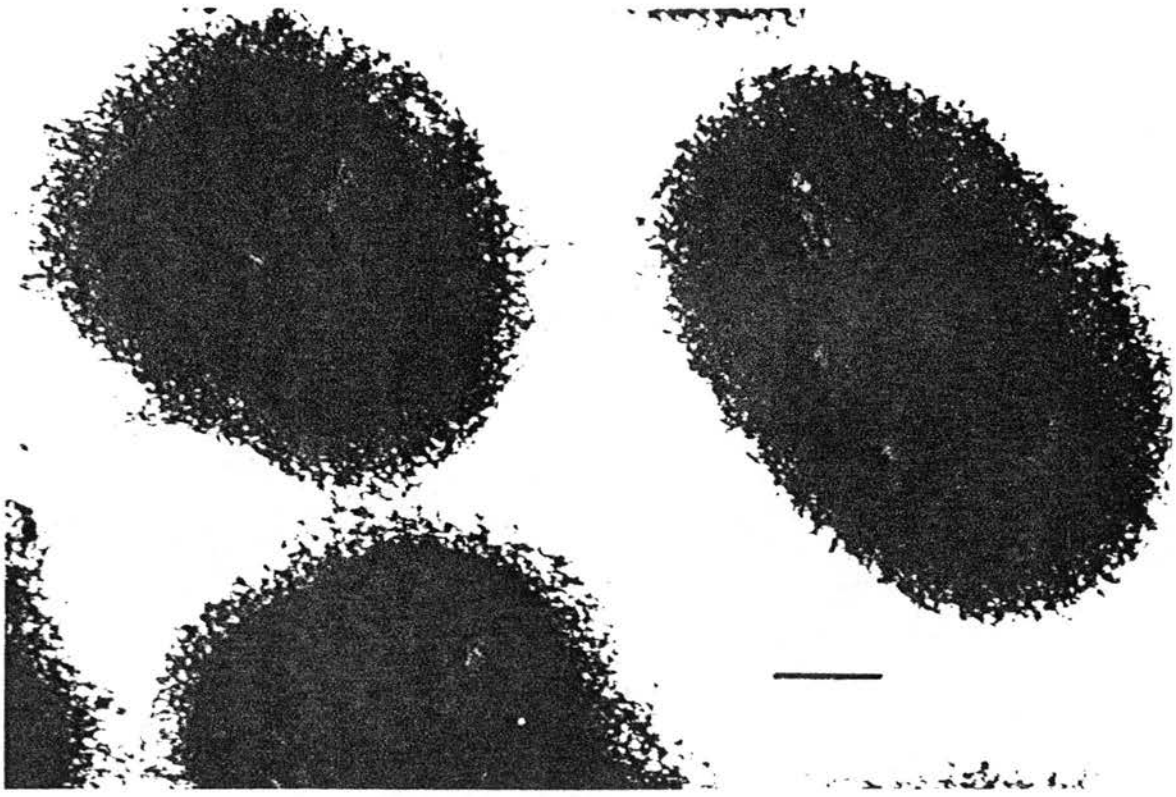


Fig. 13.1 Electron micrograph of a capsulate *Klebsiella* sp stained with ruthenium red. Bar marker = 0.2 μm. (Photograph kindly supplied by Dr J. R. W. Govan)

invasive of bacteria is *Salmonella* Typhi. In this species the most important virulence determinant is the Vi antigen. This capsular antigen, which has a unit structure of partially O-acetylated, α-1,4-linked *N*-acetyl-galactosaminuronic acid monomers, is important in invasion. The invasive, but non-capsulate, shigellae and enteroinvasive *E. coli* (EIEC) have unusual acidic O-polysaccharides which are proposed as functional analogues of the acidic capsule and as being important in the mechanism of invasion.

The capsule of *Bacteroides fragilis* and possibly of other *Bacteroides* spp. is said to have a role in inhibiting the phagocytosis and intracellular killing of facultative anaerobes (predominantly *E. coli*) in mixed anaerobic and aerobic infections. This conclusion is from a series of observations, both *in vitro* (Ingham *et al.* 1981, Reid and Patrick 1984, Jones and Gemmell 1986, Patrick 1988) and *in vivo* (Onderdonk *et al.* 1976, 1977, Kelly 1984, Patrick *et al.* 1986) that *Bact. fragilis* acts synergistically with the aerobe. The mechanism is obviously multifactorial, with LPS, proteins, metabolites, chemotaxis of neutrophils and the redox potential of the system all possibly having an influence. It appears that a surface polymer is likely to be involved in the process; but, as the experiments *in vitro* were performed with capsular polysaccharide contaminated with LPS and vice versa, it is not possible to be precise as to the mechanisms involved (Poxton and Brown 1986).

Lipopolysaccharides

It is considered that, more than other cell-surface components, lipopolysaccharide (LPS, endotoxin) is associated with virulence. This amphipathic molecule, which is anchored in the outer membrane of the gram-negative bacterium and forms an essential constituent of the cell envelope, confers on the organism a wide range of biological properties. Much of the work on the structure–function relationships of LPS has originated in laboratories in Freiburg and Borstel in West Germany. The recent review by Brade *et al.* (1988) is recommended as a comprehensive summary of the current knowledge in this field.

Structure In structure, LPS consists of two main regions: a **hydrophobic glycolipid** – the lipid A – and a **hydrophilic polysaccharide**. In many bacteria with a smooth-form LPS the polysaccharide can be subdivided into the core oligosaccharide and the O-polysaccharide or O-antigen. Rough mutants lack the O-polysaccharide. Some bacteria (e.g. *Neisseria* spp.) possess a naturally rough-form LPS which is sometimes referred to as a lipo-oligosaccharide. The smooth/rough structure of LPS is summarized diagrammatically in Fig. 13.2.

The lipid A is the toxic part of the molecule. In *E. coli* it consists of a β-1,6-linked glucosaminyl-glucosamine disaccharide backbone which is substituted with phos-

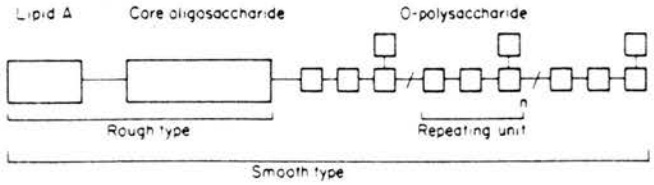


Fig. 13.2 Diagrammatic representation of lipopolysaccharide

phate groups at the l-position of the reducing end and at the 4-position of the non-reducing sugar. Each sugar monomer is substituted at the 2- and 3-positions with 3-hydroxymyristic acid residues through amide and ester linkages respectively. The fatty acids on the non-reducing sugar are themselves substituted with lauric and myristic acid. Figure 13.3 shows the structure of the lipid A molecule of *E. coli*. This structure is more or less common to all LPS molecules that are endotoxic, the variations being in the length of the substituted fatty acid residues, the type and distribution of the substituents of the fatty acids and the additional substituents of the phosphoryl residues.

The structure of the core oligosaccharide consists of 11 or so monosaccharide units. The inner region of the core (i.e. the part proximal to the lipid A) is characterized by being made up of the unusual sugars 3-deoxy-D-*manno*-2-octulosonic acid (KDO) and L-*glycero*- or D-*glycero*-D-*manno*-heptose (heptose) together with phosphate and ethanolamine. This inner region of the core is largely conserved in a wide range of species. The outer part, consisting typically of glucose, galactose and glucosamine is where a limited amount of variation between and within species occurs. For example, there are probably about six core-types for *E. coli* and only one for *Salmonella*. Examples of core types are shown in Table 13.3; it should be stressed, however, that the structures presented here are the best estimates from the data available. Microheterogeneity certainly exists in the core, especially in the non-sugar substituents. It is likely that modifications of the structures will be made in the light of results obtained from the increasing use of high-resolution nuclear magnetic resonance spectrometric analyses. In some species (e.g. *Vibrio cholerae*), it is often difficult to demonstrate the presence of KDO because of unusual substitution or linkage patterns; in other gram-negative bacteria (e.g. a strain of *Acinetobacter calcoaceticus*) it appears to be absent, but an analogous sugar is present (Brade *et al.* 1988).

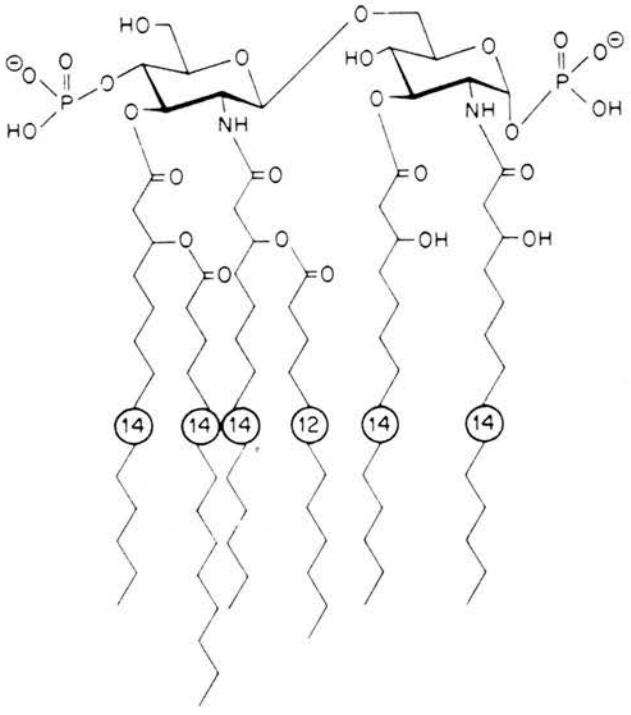


Fig. 13.3 The structure of lipid A from *Escherichia coli*. (Adapted, with permission, from Brade *et al.* (1988))

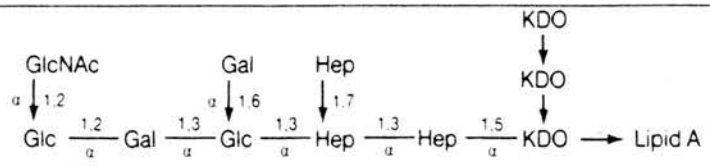
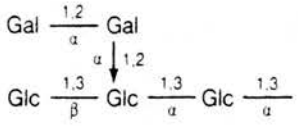
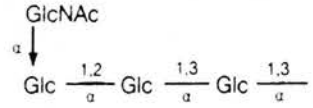
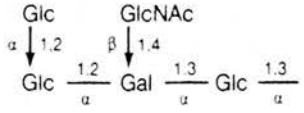
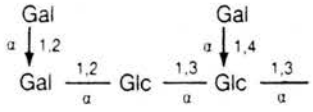
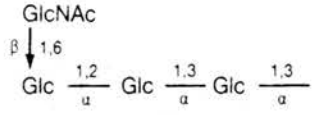
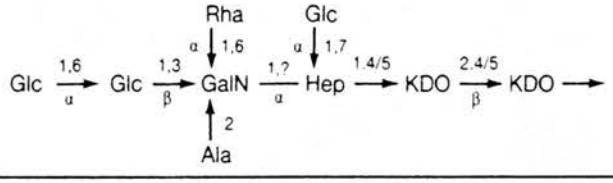
It is in the O-polysaccharide where variation can be almost infinite. This is the part of the molecule in which the O-serotype specificity resides. The structure consists typically of long chains of repeating units of oligosaccharides, usually made up of three or four monomers. There are over 160 serotypes in *E. coli* whereas in *Salmonella* there are over 60 types and in *Ps. aeruginosa* only 17. Examples of the repeating unit structure of some O-polysaccharides are shown in Table 13.4.

A feature of the O-polysaccharide that has become apparent in recent years is its considerable variability in chain length. A smooth bacterium possesses a range of O chain lengths ranging from unsubstituted core oligosaccharide to 30 or more repeating units. This is best demonstrated by analysis of LPS by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with silver (Hancock and Poxton 1988). An example of this is shown in Fig. 13.4. This variability in chain length influences the biological properties of the LPS.

Certain serotypes of *E. coli* (e.g. 0111) produce some of their O-polysaccharide in a form which is not linked to core and lipid A, but is polymerized to form a capsular polysaccharide resembling a K antigen. This has been investigated in detail by Peterson and McGroarty (1985) by separation of LPS fractions by gel filtration followed by SDS-PAGE.

Biological activities The toxic part of the LPS molecule resides in the lipid A region. This part alone can be shown to express all the toxic biological properties of **endotoxin**. The core and O-polysaccharide component, however, have a marked influence on the fate of LPS in the host (Fig. 13.5) and on the mechanisms of virulence and pathogenicity of the bacterium. Table 13.5 summarizes the biological properties of LPS. In the host, the culmination of the effect of LPS is **endotoxic shock**, the main features of which are hypotension, a fall in cardiac output and disseminated intravascular coagulation, which lead to death (Morrison and Ryan 1987). Some bacteria (e.g. *Bact. fragilis*) possess an LPS of low endotoxicity and this is reflected in the structure of lipid A, especially the fatty acid composition and phosphate substitution (Weintraub *et al.* 1985).

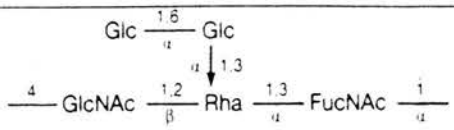
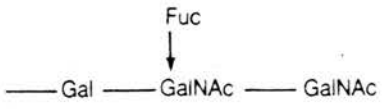
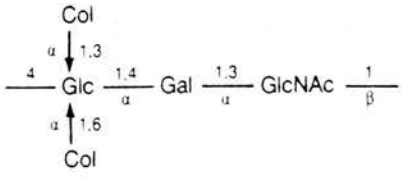
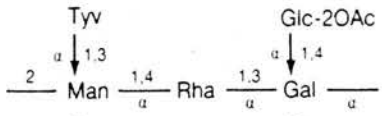
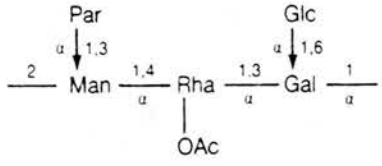
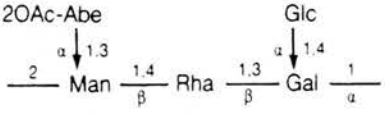
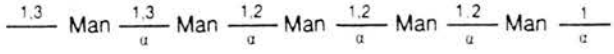
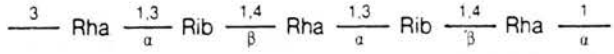
Table 13.3 Lipopolysaccharide core oligosaccharides

Core type	Structure
Salmonella	
Escherichia coli R1	
E. coli R2	
E. coli R3	
E. coli R4	
E. coli K-12	
Pseudomonas aeruginosa	

Abbreviations: Ac, acetyl; Ala, alanine; Gal, galactose; GalNAcUA, *N*-acetylgalactosaminuronic acid; GalN, galactosamine; GalU, galacturonic acid; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Hep, heptose; KDO, 3-deoxy-D-*manno*-2-octulosonic acid; ManNAc-1-P, *N*-acetylmannosamine phosphate; NeuNAc, *N*-actylneuraminic acid; Rha, rhamnose; Rib, ribose; Rib-P, ribitol phosphate.
Data from Rowe and Meadow (1983) and Jann and Jann (1984).

The exact mechanisms by which LPS harms the host are by no means clearly understood, but the structure of lipid A in relation to fatty acid and phosphate substitution is of great importance. As a virulence determinant the form of the LPS greatly affects the potential of the bacterium to harm the host. Rough, isogenic mutants are usually 1000 times or so less virulent than their smooth parents, and even minor changes in the composition of the sugar substituents of O-polysaccharide can markedly influence the virulence for mice. As described previously for capsular polysaccharide, the long O-polysaccharide chains confer on the bacterium resistance to the lytic action of serum and protects against engulfment by phagocytes. LPS is a good activator of complement by both the alternative pathway – independent of antibody – and the classical pathway (Morrison and Kline 1977). It is presumed that the long chains protect the inner region of the LPS which is associated with the bacterial outer membrane (Tomas *et al.* 1988). Anti-O antibodies may, however, be bactericidal. Furthermore, the longer the polysaccharides, the more hydrophilic is the cell. It is likely that the degree of heterogeneity in chain length may be of paramount importance to the balance of the interaction between the host defence system and the gram-negative bacterium: the greater the preponderance of long chains, the greater the virulence. This factor is no doubt influenced by the growth environment of the bacterium, so predictions based on cells grown *in vivo* may be false. *N. gonorrhoeae* with naturally rough LPS (sometimes termed lipo-oligosaccharide) is usually sen-

Table 13.4 Examples of O-polysaccharide repeating units

Species (serotype)	Structure
Escherichia E. coli (04)	
E. coli (086)	
E. coli (0111)	
Salmonella Typhi	
Salm. Paratyphi A	
Salm. Typhimurium	
Klebsiella (03)	
Klebsiella (010)	

Abbreviations: Abe, abequose (3,6-dideoxy-D-galactose); Ac, acetyl; Ala, alanine; Col, colitose (3,6-dideoxy-L-galactose); Gal, galactose; Fuc, fucose; FucN, fucosamine; GalNAcUA, *N*-acetylgalactosaminuronic acid; GalN, galactosamine; GalU, galacturonic acid; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Hep, heptose; KDO, 3-deoxy-D-*manno*-2-octulosonic acid; Man, mannose; ManNAc-1-P, *N*-acetylmannosamine phosphate; Par, paratose (3,6-dideoxy-D-glucose); NeuNAc, *N*-acetylneuraminic acid; Rha, rhamnose; Rib, ribose; Rib-P, ribitol phosphate; Tyv, tyvelose (3,6-dideoxy-D-mannose)

Data from Jann and Jann (1984)

sitive to the lytic action of serum. However, strains isolated from disseminated gonococcal infections are serum-resistant. In these strains the LPS appears to be altered in an unknown way (Stephens and Shafer 1987).

In the previous section on capsules, it was noted that the small group of serotypes of shigellae and enteroinvasive *E. coli* characterized by possessing acidic O-polysaccharides act in a manner analogous to that of invasive bacteria with acidic capsules, the acidic surface structure being necessary for the mechanism of invasion. Furthermore, in a way equally poorly understood, certain LPS types (serotypes) are associated with other pathogenic mechanisms. For example, the enterotoxigenic *E. coli* (ETEC) are confined to a small number of serotypes, the enteropathogenic *E. coli* (EPEC) belong to another small group, and the same is true of enterohaemorrhagic *E. coli* (see below). There is thus a circumstantial link between surface chemistry and pathogenicity. Whether this has anything to do with recognition of host tissue or perhaps susceptibility to infection by a bacteriophage or plasmid (in the way that other virulence determinants are phage or plasmid-encoded) is not known.

Teichoic acids and related polymers

Structure The surface of the gram-positive bacterium typically has two structurally related, negatively charged components. One is covalently linked to the peptidoglycan and is usually referred to as the teichoic acid or the secondary (or accessory) cell wall polymer. Under conditions of phosphorus limitation this can be replaced by teichuronic acid. The other is anchored in the cytoplasmic membrane and protrudes through the wall. This

Fig. 13.4 The heterogeneity of lipopolysaccharide (LPS) demonstrated by polyacrylamide gel electrophoresis and silver staining. Samples of LPS from six blood-culture isolates prepared by the proteinase K method and run on a 12% polyacrylamide gel. Track 1, *Escherichia coli* 075; track 2, *E. coli* 018; tracks 3 and 4, *Pseudomonas aeruginosa*; track 5, *E. coli* 01; track 6, *E. coli* 06. See Hancock and Poxton (1988) for technical details. (Photograph kindly supplied by Dr A. P. Gibb)

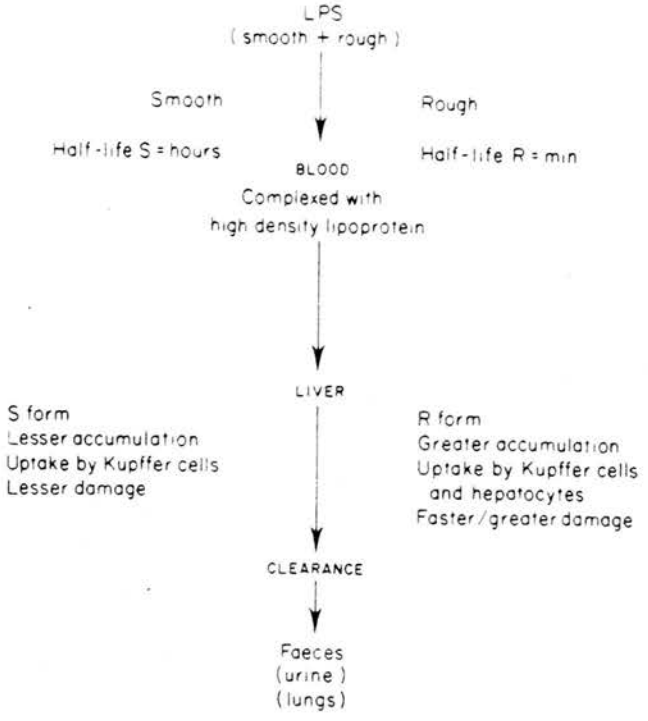


Fig. 13.5 The fate of smooth and rough lipopolysaccharide (LPS) in the host. (Data from Freudenberg and Galanos (1986))

is the lipo- or membrane-teichoic acid, or lipocarbohydrate. The terminology is complex because a teichoic acid is, by definition, a polymer containing ribitol phosphate or glycerol phosphate with phosphodiester linkages, usually with hexose and D-alanine substituents (Baddiley 1972). The lipoteichoic acids are usually based on glycerol phosphate and have at their terminus a glycolipid. Teichoic acids in this strict sense are not found in all gram-positive bacteria, but there is usually a functionally analogous molecule. If phosphate is absent, acidic sugars can contribute a negative charge, and the polyol is sometimes replaced by a hexose. Examples of this family of molecules are shown in Fig. 13.6.

Biological activities To discuss the secondary wall polymers first: they are not well known as virulence factors. Functionally their most important role is possibly in sequestration of divalent cations. They are immunogenic and are the group antigens in many streptococci, staphylococci and lactobacilli. Some may have a role in inflammation, an example being the C substance (C-teichoic acid) of *Str. pneumoniae* which binds to the acute phase (C-reactive) protein.

The lipocarbohydrates have been proposed as analogues in gram-positive bacteria of the LPS in gram-negative organisms. Many of the endotoxic properties of LPS are said to be mediated by lipoteichoic acids, but this is debatable; contamination with LPS is thought to

Table 13.5 The biological properties of lipopolysaccharide

Pyrogenic for man and rabbits
Lowers temperature in mice
Lethal toxin
Activation of complement
Activation of clotting cascades
Limulus amoebocyte lysate gelation
B-cell mitogen (T-independent)
Local Shwartzman reaction
Induction of tumour necrosis factor
Induction of interleukin-1
Induction of colony-stimulating factor
Induction of prostaglandins and leukotrienes
Induction of non-specific resistance to infection

Data from Morrison and Ryan (1987) and Brade *et al.* (1988)

be responsible for much of the confusion. Lipoteichoic acids have a role in channelling divalent cations to the cytoplasmic membrane and are involved in cell division as substrates for autolysins. They are probably anti-complementary and are phage receptors. They may be responsible for cross-reactions between a range of gram-positive bacteria; some false positive results in the Kahn test for syphilis may be due to the cross-reaction between cardiolipin and the phosphorylglycerol phosphate grouping. Perhaps the major role for lipoteichoic acids in virulence is in adhesion. One proposal is that lipoteichoic

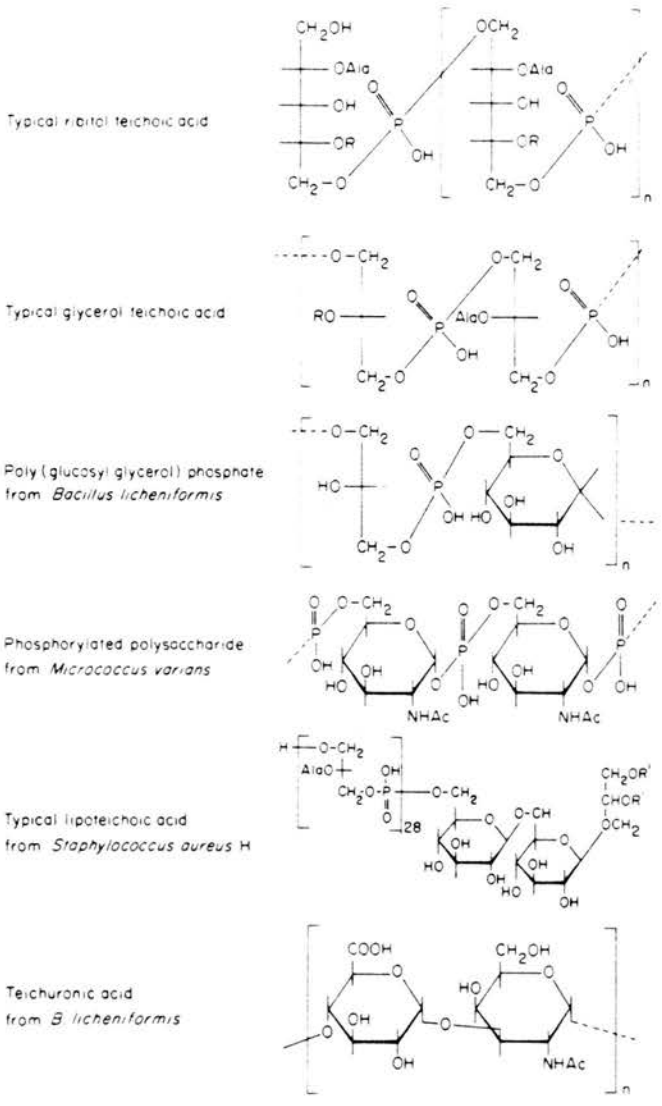


Fig. 13.6 Structures of some teichoic acids and related polymers. n, 30–40. R, sugar substitute. R', fatty acid. (Compiled from Duckworth 1977).

acids and deacylated lipoteichoic acids interlink through cationic bridges at their phosphate groups to bind *Str. mutans* to the tooth surface. Another is that adhesion of *Str. pyogenes* to the epithelium of the pharynx involves a combination of M protein (see later) and lipoteichoic acid in a fibrillar meshwork, which binds to fibronectin on the host cell surface through the lipid moiety of the lipoteichoic acid (Beachey and Courtney 1987).

Protein virulence factors

Proteins associated with the bacterial cell surface are in two main forms; those that form appendages discernible in the electron microscope – the **fimbriae** (or **pili**) and **flagella** – and those present on the surface as a **layer**, which often form a regular array, or are embedded in or are associated with the gram-negative outer membrane. Many of these proteins have well defined roles in virulence, most commonly involving adherence. For a recent review on adhesins of the bacterial cell envelope the reader is referred to Old (1988).

Fimbriae (pili)

These synonymous terms describe the filamentous protein appendages found on a variety of bacteria. In this chapter the term 'fimbriae' will be used for all such appendages with the exception of those of the gonococcus in which they are conventionally referred to as pili. The structurally similar, but functionally different, sex pilus used in conjugation (see Chapters 2 and 6) is not described further here.

Fimbriae of Enterobacteriaceae It is in the Enterobacteriaceae that fimbriae are best characterized. The pioneering work of Duguid more than 30 years ago established the presence of fimbriae by agglutination of red blood cells from a range of species. This fimbria-mediated haemagglutination could be resistant or sensitive to blocking by D-mannose (Duguid *et al.* 1955, 1966). The mannose-sensitive (MS) fimbriae are usually termed common or (type 1) fimbriae, and are widespread in most species of enterobacteria. Structurally they are made up of about 1000 protein subunits of mol. wt 17000 which form a straight, tubular structure about 1 μm in length and 7–8 nm in diameter with a central hole (Brinton 1965). These fimbriae bind to a wide range of cell types from many different species. Antigenically they do not show much cross-reaction with each other and are even distinct within a species. It is still, however, usual to group them together as they react with an α-D-mannosyl-containing receptor. Recent evidence tends to suggest that this approach is perhaps too simplistic (Old 1988). Ørskov and Ørskov (1983) studied the serology of *E. coli* fimbriae. Expression of fimbriae by bacteria is dependent on cultural conditions, and temperatures must be above 20°. Type 1 fimbriae are optimally produced at 37° in static broth cultures. Some of the MR fimbriae and gonococcal pili require solid medium for their expression. Generally, the expression of fimbriae is better on minimal than on rich media.

Until recently it was thought that type 1 fimbriae were not virulence determinants. It was in fact considered that their possession is detrimental to the bacterium. They promote better binding to polymorphonuclear leucocytes and are therefore better phagocytosed; moreover, as potential urinary tract pathogens, they bind to Tamm–Horsfall glycoprotein (in urinary mucus) and are flushed out with the urine (Ørskov *et al.* 1980). This concept has, however, been challenged by Kuriyama and Silverblatt (1986), who have shown that type 1 fimbriate bacteria are resistant to phagocytosis when coated with the Tamm–Horsfall glycoprotein. Furthermore, type 1 fimbriate *E. coli* colonize the epithelium of the bladder better than non-fimbriate types, and receptor analogues protect against experimental infections (Old 1988). There remains much to be clarified in the role of these common fimbriae in urinary tract infections.

Table 13.6 Strains of *Escherichia coli* that cause diarrhoea

Category of *E. coli*	Common O serogroups	Virulence factors
Enterotoxigenic (ETEC)	06, 08, 011, 015, 020, 025, 027, 063, 078, 080, 085, 0115, 0128, 0139, 0148, 0149, 0153, 0159, 0167	Heat-labile (LT) and heat-stable (ST) toxins Fimbrial adhesion
Enteropathogenic (EPEC)	020, 044, 055, 086, 0111, 0114, 0119, 0125, 0126, 0127, 0128, 0142, 0158	Shiga-like toxin Outer membrane protein adhesins
Enteroinvasive (EIEC)	028, 029, 0112, 0124, 0136, 0143, 0144, 0152, 0164, 0167	Invasion of epithelium Shiga-like toxin
Enterohaemorrhagic (EHEC)	026, 0111, 0157	Shiga-like toxin Fimbrial adhesins

Data from Orskov (1978), Levine (1987) and Law (1988)

The role of another fimbria, the mannose-resistant P fimbria, has a much less debatable role in urinary tract infections. More than 90% of *E. coli* strains isolated from non-obstructive pyelonephritis in young children possess P fimbriae. They were so named because they bind to the P blood group antigen. P fimbriae recognize and can bind to a D-gal-α-(1,4)-D-gal structure on epithelial cells as well as on red cells (Kallenius *et al.* 1980). This structure is abundant in the glycolipids of the upper ureter and kidney. It has been proposed that secretor status may influence individual susceptibility to urinary tract infections. Women with recurrent urinary tract infections and scarring of the kidney tend to be non-secretors (Lomberg *et al.* 1986). It has been suggested that non-secretors may have more receptors available for binding P-fimbriate bacteria.

Similarly, other fimbriae designated X, S and M fimbriae have been described and their receptors identified; e.g. the S fimbriae bind to vascular endothelium through a NeuAc-α-(2–3)-gal, β-(1–3)-galactosamine receptor and may be an important virulence determinant in strains of *E. coli* found in neonatal meningitis. A review by Lindberg *et al.* (1988) on protein–carbohydrate interactions in bacterial pathogenicity is recommended for further information on receptors for fimbriae.

Another well known group of fimbriae and fimbria-like structures is found on *E. coli* strains causing diarrhoea in man and domestic animals (reviewed recently by Levine 1987). The types of *E. coli* recognized as causing diarrhoea are listed in Table 13.6. However, not all of these types produce fimbrial adhesins. Classically, fimbriae are associated with the enterotoxigenic *E. coli* (ETEC) and the adhesins are often referred to as colonization factors. They include the mannose-resistant K88, K99, 987P and F41 antigens that cause diarrhoea in animals, and CFA I, CFA II and E8775 found in human strains. In structure, some (e.g. CFA I and 987P) resemble common fimbriae, whereas others (e.g. K88 and K99) appear as more flexible and much thinner fibrils with a diameter of 2–3 nm. These are sometimes referred to as fibrillae. The CFA/II and E8775 antigens consist of families of adhesins. The CFA/II strains carry CS1, CS2 and CS3 antigens (Smyth 1982). Almost all strains carrry CS3, usually with CS1 or CS2, CS1 and CS2 are like the typical type 1 fimbriae, and CS3 is of the thin, flexible type. Similarly, E8775 strains carry combinations for CS4 and CS5 (rigid) and CS6 (flexible) types.

The possession of different types of fimbriae is closely related to the O-serotype of the organism. ETEC strains belong to a small group of O-serotypes and it is probable that all these serotypes possess a fimbrial adhesin. The role of fimbriae in causing diarrhoea is to localize the pathogen on the brush border of the mucosa of the anterior ileum, usually in the first metre or so. Here the organisms deliver the other major virulence factor of ETEC strains: a heat-labile (LT) enterotoxin, a heat-stable (ST) enterotoxin, or both. The enterotoxins and adhesin are encoded on the same virulence plasmid. These features of ETEC are described in detail later.

The receptor for the colonization factor is usually found in a narrow range of hosts. Some organisms tend to bind only to the ileum of man, and others only to that of certain animals. For example, *E. coli* carrying the K88 antigen seems to be found only adhering to the mucosa of porcine ileum, and K99 strains are found usually in calves and lambs. After experimental inoculation, K88 and K99 strains do not produce diarrhoea in calves and pigs respectively. Even within a host species, certain individuals are resistant to infection. In piglets there are five recognized phenotypes of brush border, one of which is totally resistant to colonization by K88. This resistance is transferred genetically by mendelian inheritance. The chemical basis of the host receptors is not well understood. The age of the host is also important in susceptibility to diarrhoea. Calves are susceptible to K99 only during the first 2 days after birth, whereas piglets are susceptible to 987P strains only in the neonatal period but to K88 at two different ages: when younger than 6 days and during the post-weaning period (3–9 weeks). A mechanism for resistance to 987P after the neonatal period has been proposed by Dean *et al.* (1989). The brush border expresses the 987P receptor in both neonatal and older pigs, but in the latter it is released into the lumen of the intestine and facilitates bacterial clearance. For a detailed review of the host specificity of fimbrial

adhesins in diarrhoeal disease, see Gaastra and de Graaf (1982).

Of the other *E. coli* strains which cause diarrhoea, only the uncommon enterohaemorrhagic (EHEC) strains are recognized as possessing fimbriae. In man, *E. coli* 0157:H7 is the serotype associated with the bloody diarrhoea of haemorrhagic colitis. This syndrome is distinguished from dysentery by the lack of fever and by the copious volumes of stool. The strain is also associated with haemolytic uraemic syndrome which, together with haemorrhagic colitis, is being increasingly reported in North America (Levine 1987). The strains are characterized as possessing a plasmid of mol. wt 60×10^6 which codes for the fimbriae involved in binding the bacteria to cells.

Fimbriae of *Neisseria* The last type of fimbria to be described in detail in this chapter is that produced by *N. gonorrhoeae*. The convention is that they are termed 'pili' rather than 'fimbriae', and this will be observed here. They are similar in appearance to the type 1 fimbriae of enterobacteria.

Fresh isolates of *N. gonorrhoeae* are invariably piliate, but on repeated subculture on solid medium they lose their pili and undergo phase variation, the non-piliate forms giving rise to a different colonial morphology (Brinton 1965). Differences in colonial opacity can also result from variations in outer membrane proteins but this point will be discussed later. Like the fimbriae of enterobacteria, gonococcal pili are made up of identical subunits of pilin, with a mol. wt of c. 20000. At least 12 antigenic variants of pili have been recognized in gonococci (Swanson and Berrera 1983) but a single strain which might have the capacity to produce up to seven types possesses only a single type at any one time. On genetic evidence there is potential for the production of many more types than 12 (Haas and Meyer 1986). The variant pili from a single strain differ slightly in the molecular mass of their pilin subunits and in their antigenic cross-reactivity. When antibodies were raised to four different types of pilus isolated from a single strain, there was only 10–20% cross-reactivity between heterologous strains (Virji *et al.* 1982). These antigenic variations also occur *in vivo*, as demonstrated by analysing sequential isolates from a single patient (Heckels 1984).

The molecular basis for this variation has been determined by sequencing the pilin subunit. The N-terminus of the molecule consists of a highly conserved region of about 50 amino acids. This region is not immunogenic. The C-terminus consists of a highly variable region, and it is this portion that is immunodominant and gives rise to the antigenic variation (Hagblom *et al.* 1985). This finding makes the possibility of an antipilus vaccine unlikely.

The function of the pili of the gonococcus is mainly one of **adherence**. They stick to a wide range of cell types. It is thought, however, that they are also **antiphagocytic**, preventing binding to polymorphonuclear leucocytes. Certain of the outer membrane proteins are also involved in adherence and will be discussed later.

The closely related *N. meningitidis* possesses pili structurally and antigenically related to those of the gonococcus (Stephens *et al.* 1985). The fimbriae of *Pseudomonas* and *Moraxella* spp. are also related to the pili of *Neisseria* (Froholm and Sletten 1977, Saastry *et al.* 1983).

Fimbriae of other bacteria The fimbriae (pili) described above are the best known examples of their role as virulence factors. There are, however, many other examples in bacteria (usually gram-negative) that possess fimbriae. In *Haemophilus influenzae*, fimbriae are involved, possibly in conjunction with capsules, in adherence. For a recent review of the mechanisms of pathogenicity of *H. influenzae* investigated from a genetic point of view, see Wilson and Moxon (1988). Fimbriae are also found in *Bordetella pertussis*, *Ps. aeruginosa*, certain *Bacteroides* spp. and *Corynebacterium diphtheriae*, and are putatively involved in adherence.

Flagella

Structure These whip-like appendages are the components of the bacterial cell responsible for movement. Not all bacteria possess the ability to move. When present, however, the arrangement of the flagella on the cell is species-dependent. Some species possess a single polar flagellum (monotrichous), whereas others have polar tufts (lopotrichous) and in some the surface is completely covered with flagella (peritrichous). The flagellum is primarily a long filament made up of strands of flagellin subunits of molecular weights in the range 33000–66000. The final structure is a fairly rigid tube of 10–20 μm in length and 12–20 nm in diameter with a hole of diameter c.3 nm in its centre. Some bacteria have a sheathed flagellum, e.g. those of *Vibrio* and *Pseudomonas* spp. The base of the flagellum is anchored into the bacterial envelope by means of a hook-like organelle attached to a basal body. The structure and function of the bacterial flagellum were reviewed by Doetsch and Sjøblad (1980); see also Chapter 2.

Biological activities As antigens, the flagella are usually strongly immunogenic. They are the classic H antigens of the Enterobacteriaceae. As candidate virulence factors they are, however, probably not of great significance in many motile organisms. This is despite their potential for aiding the pathogen to traverse mucus and to penetrate cells, although their role in propelling bacteria towards chemoattractants may be of importance.

It is in *V. cholerae* that flagella are best known as a putative virulence factor. The main virulence factor of this pathogen is cholera toxin which is described later, but the penetration of the mucus blanket of the small intestine is a necessary first step, and this might be followed by adherence and localisation at the epithelial surface. Attridge and Rowley (1983) showed that a flagellum is necessary for attachment *in vitro*; however, a *functional* flagellum is not required, as flagellate, nonmotile organisms bind to cells as well as flagellate, motile

organisms. Furthermore, in an infant mouse model of cholera the flagellum enhanced virulence by facilitating the initial colonization of the ileum. Similar observations have been made for *Campylobacter jejuni* but in this instance it is acknowledged that many other adhesins may play a part (Walker *et al.* 1986).

It has been reported (Weinstein *et al.* 1984) that the flagella of *Salm.* Typhimurium help the organism to survive in murine macrophages by protecting it from intracellular killing or by enhancing its ability to multiply.

The axial filaments found in some of the spiral organisms, *viz. Treponema, Leptospira* and *Borrelia* spp., resemble flagella in structure. In some cases they appear to be protective antigens, but their role in virulence is uncertain.

Outer membrane proteins

Structure The outer membrane (OM) of the gram-negative bacterium appears as a typical bilayer in the electron microscope. It is made up of phospholipids, protein and, in its outer leaflet, lipopolysaccharide. When analysed by PAGE the number of proteins appears initially to be rather small because there are only a few dominant major OM proteins. There are, however, many minor polypeptides. Some of the major proteins can form transmembrane channels (porins) or may be part of either the inner or the outer leaflet. The functions of OM proteins are numerous and include ion binding, transport systems, a few enzymatic functions, receptors for phage and bacteriocins, and adhesins. The structure of the OM is described briefly in Chapter 2; for more detailed accounts, see Vaara and Nikaido (1984) or Nikaido and Vaara (1985) and, for OM proteins, Osborn and Wu (1980).

The LPS component of the OM was described earlier in this chapter, but here it should be stressed that LPS and OM proteins are extremely closely associated and the role of one might be closely influenced by the other. In the isolation and analysis of OM proteins it is extremely difficult to obtain them free of LPS (Poxton *et al.* 1985).

Biological activities Perhaps the function of OM proteins as **adhesins** is the best recognized of their roles in virulence, and, as for the other classic adhesins, the fimbriae, it is in diarrhoea-causing *E. coli* and the gonococcus that they have been studied most. The types of *E. coli* that cause disease are listed in Table 13.6, and, as described earlier, the ETEC and EHEC are known to have fimbriae as their major adhesins. In the EPEC strains, however, an OM protein is probably an adhesin. The identification of this adhesin followed the recognition by Cravioto *et al.* (1979) of a plasmid of mol. wt c. 60×10^6 which encoded for adherence to HEp-2 cells and was termed the EPEC adherence factor (EAF). This plasmid was subsequently shown to code for an OM protein of mol. wt 94000 (Levine *et al.* 1985) which is found in all the important EPEC serogroups (Levine 1987). A DNA probe has been developed for the detection of the EAF (Nataro *et al.* 1985). A critical review of the virulence factors of EPEC strains has been made by Law (1988).

The EIEC and shigellae are characterized as invasive gut pathogens. After entering the epithelial cells of the colon they multiply and kill the cell. A plasmid of mol. wt 140×10^6 appears to code for several OM proteins proposed as being involved in the invasion process (Harris *et al.* 1982, Hale *et al.* 1983). Furthermore, in other invasive organisms (e.g. *Yersinia* spp.) several plasmid-coded OM proteins – along with other factors – have been proposed as important in invasion. The mechanism of invasion is not clearly understood. Apart from the initial attachment of the bacterium to the host cell, the OM proteins may also be involved in survival and multiplication of the bacterium within the host. For a recent review on enteroinvasive bacteria, see Sansonetti *et al.* (1988).

The observation that non-piliated gonococci can still bind to cells led to the identification of a second adhesin, the OM protein II, or PII. Colonial variants, differing in opacity, are commonly observed when a single strain is grown on solid medium. This variation is due to variation in expression of PII. A total repertoire of about six different types of PII is found in each strain (Swanson 1978). In common with the pili of the gonococcus, antigenic variation of PII can occur in a single strain during the course of a disease in a single patient (Schwalbe *et al.* 1985).

Another class of OM proteins recognized as important virulence factors are the iron-regulated membrane proteins. These are known to be expressed under conditions of iron limitation by a number of bacteria, and more are being recognized. They act as virulence determinants by being able to compete for iron in the iron-depleted environment of the host. This feature will be covered in more detail later in the sections of phage-associated virulence and on expression of virulence determinants.

Other surface proteins

The other proteins found on the surfaces of bacteria include the crystalline surface arrays found on many gram-positive and gram-negative bacteria and were described briefly in Chapter 2. The species most investigated are not pathogenic and therefore no role in virulence has been proposed for these proteins. There are, however, certain proteins on gram-positive bacteria – notably the **M-protein** of *Str. pyogenes* – that have a significant role in virulence; protein A of *Staphylococcus aureus* may also play some part in enhancing infection.

Streptococcal M protein

The M protein along with the hyaluronic acid capsule and the lipoteichoic acid are recognized as the surface virulence determinants of *Str. pyogenes*. There are over 80 different serological types of M protein and they are used in the serological classification of group A streptococci. M protein exists as a fibrillar surface layer and each molecule consists of two α-helical protein chains in a coiled-coil superstructure anchored to the cytoplasmic membrane at its C-terminus. As described earlier, in the

section on lipoteichoic acids, it has been proposed that the two polymers combine to form an adhesin. The M protein also protects the organism from recognition and ingestion by phagocytic cells, but this can be overcome by opsonic anti-M immunoglobulins (Lancefield 1962). It is also known that M protein binds the C3 component of complement, but this binding is blocked if the bacterium first binds fibrinogen from plasma (Whitnack and Beachey 1985). The hyaluronic acid capsule, which has earlier been described as a camouflage mechanism for the bacterium, is also said to bind fibrinogen and thus to block C3 binding (LeBouef *et al.* 1986). By cloning *Str. pyogenes* M protein into *Str. sanguis*, Poirier *et al.* (1989) have reinforced the suggestion that binding of M protein to fibrinogen allows evasion of the host defence system. An alternative explanation of the antiphagocytic activity of M protein was put forward by Fischetti *et al.* (1988), who suggested that, in serum, M protein binds factor H, the serum control protein of the alternative complement pathway, and so prevents phagocytosis by depleting C3b on the streptococcal surface. For details of the complement system, see Chapter 16.

Protein A of *S. aureus* and a group of similar proteins (e.g. protein G) found on other gram-positive bacteria have the property of binding the Fc fragment of immunoglobulins. Protein A is found in most strains of *S. aureus* but the Cowan strain is usually used for its preparation. Molecules of this class have become of great importance in recent years because of their use in the isolation and purification of immunoglobulins. As virulence factors, most of their potential roles are hypothetical. The binding of immunoglobulins by their 'wrong ends' may be an antiphagocytic property. Furthermore, binding of the Fc region may result in the release of inflammatory agents.

The association of plasmids and bacteriophage with virulence

As mentioned earlier in this Chapter, there is often a close association between virulence determinants and the possession of plasmids; in fact, the more determinants investigated, the more are shown to be plasmid-mediated. These plasmids are referred to as **virulence plasmids** or *vir* plasmids. **Bacteriophages**, as prophages, are also known to code for virulence determinants in a few well recognized instances. (See also 'Virulence factors' in Chapter 6.)

Plasmids

In 1980, Elwell and Shipley (1980) reviewed the plasmid-mediated factors associated with virulence. Since then several advances have been made. The gram-negative bacteria, especially *E. coli*, other enterobacteria and *Yersinia* spp., have been the most studied. In *E. coli* causing diarrhoea, both the enterotoxin genes and the colonization factor genes are coded for by plasmids (see earlier in this Chapter and Levine (1987)). In the enteroinvasive *E. coli* and shigellae, the plasmid of mol. wt 140×10^6 codes for virulence and invasion, presumably by encoding for several outer membrane proteins of unknown function. Enteroinvasive *E. coli* and shigellae do not usually invade the lamina propria of the gastrointestinal tract. However, in the more invasive species – the salmonellae and yersiniae, which often become systemic invaders – plasmids have been linked both to invasion mechanisms and to resistance to the lytic action of serum. In *Salm.* Typhimurium, Vandenbosch *et al.* (1987) have shown that resistance to complement is plasmid-mediated. The phenotypic change is unknown but the LPS moiety is apparently unchanged. In *Salm.* Dublin plasmid-coded virulence is independent of the ability of the organism to cross the gut wall (Manning *et al.* 1986).

The three species of *Yersinia* pathogenic for man – *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis* – are all known to have plasmids associated with their virulence. Again the mechanisms are by no means well understood (Portnoy and Martinez 1985). The situation is complicated by the existence of multiple virulence determinants; and in *Y. pseudotuberculosis* the *inv* gene is located on the chromosome and codes for a protein of 17000 mol. wt. This has been cloned in *E. coli* and shown to permit the invasion of human cell lines (Falkow *et al.* 1987).

The best studied virulence plasmids are the ColV family of *E. coli* plasmids, which code for the small protein bacteriocin, colicin V (see 'Bacteriocins' in Chapter 5). The plasmid is invariably found in *E. coli* strains isolated from septicaemias and other invasive infections, and is strongly associated with virulence. The production of colicin V itself does not endow the host bacterium with virulence, but three other determinants coded for by the plasmid are reported to be associated with virulence (reviewed by Crosa 1984, 1987). These properties are: (1) the ability to withstand the lytic action of normal human serum, (2) the ability to adhere to mammalian cells (Darken and Savage 1987) and (3) an enhanced ability to assimilate iron. The importance of iron to pathogenic micro-organisms is well recognized (Bullen and Griffiths 1987). Free iron is present in minute quantities in tissues; most extracellular iron is complexed with iron-binding proteins such as transferrin and lactoferrin. Bacteria must obtain iron for growth and many do so with the aid of iron-chelating agents or siderophores, the best known of which is enterobactin (or enterochelin), synthesized by most of the Enterobacteriaceae (Griffiths 1983, 1987). The ColV plasmid codes for a siderophore – aerobactin, an outer membrane protein of mol. wt 74000. It is uncertain how the possession of aerobactin confers an advantage on a bacterium which can already synthesize enterobactin.

A plasmid similar to ColV and with the same properties is found in the important fish pathogen *V. anguillarum*. It codes for two outer membrane proteins.

Bacteriophage

The two best known examples of bacteriophage-associated virulence determinants are those coding for diphtheria toxin and for *Clostridium botulinum* toxin. It

has been known for many years that the β-phage of *C. diphtheriae* codes for toxin production. Crosa (1987) has summarized our present understanding of the phage-mediated expression of diphtheria toxin. The phage *tox* gene can be expressed when in a prophage genome, in a repressed genome or in replicating genomes during the vegetative phage growth. The amount of iron in the medium strongly influences the amount of toxin produced by the bacterium. Production of toxin is maximal only when the organism is growing under iron-limited conditions. The current hypothesis for the iron regulation of diphtheria toxin is that an active repressor–iron complex is formed in the presence of excess iron; this binds to the operator locus (Murphy and Bacha 1979) and switches off toxin production. *In vivo*, iron is present in such small quantities that this mechanism is inactive.

The species *Cl. botulinum* consists of eight different serotypes which produce immunologically distinct, but pharmacologically similar, toxins. It is now well recognized that types C and D, which are biochemically related, produce toxins C_1 and D only when they possess the TOX^- bacteriophage. Strains cured of the phage do not produce toxin. After reinfection with the phage, toxin production is restored and type C can be converted to D, and vice versa. Types C and D that do not produce toxin are biochemically indistinguishable from *Cl. novyi*. This species also carries a TOX^- phage which codes for α toxin. It is possible to convert *Cl. botulinum* type C to a type D or to a *Cl. novyi* by exchanging the bacteriophage (Eklund and Poysky 1981). TOX^- phages have not been identified in any of the other types of *Cl. botulinum*, but recently it has been suggested that the production of toxin G by type G strains is dependent on the possession of a single plasmid of mol. wt 81×10^6 which, coincidentally, also codes for a bacteriocin (Eklund *et al.* 1988).

The phenotypic expression of virulence determinants

It is beyond the scope of this Chapter to cover in detail this vast and topical area of medical microbiology. It has long been recognized that the environment greatly influences the properties of a pathogen. A bacterium growing in a rich nutrient medium in a laboratory must be very different from the same bacterium in an infection. How often does a fresh isolate differ in virulence from a laboratory-passaged strain? It is probably not the loss of genetic information by the pathogen which results in loss of virulence, but rather the loss of phenotypic expression, as passage in animals can often restore virulence.

Throughout this Chapter references have been made to the variation in expression of virulence determinants, e.g. the pili and PII of *N. gonorrhoeae*, the iron-regulated membrane proteins of *E. coli* and exopolysaccharide production by various species. It is probable that any virulence factor is susceptible to variation. The conditions that influence expression might include: growth temperature; growth rate; availability of nutrients; ionic strength/osmolarity; oxygen tension; pH; association in a biofilm/glycocalyx; and host factors such as complement, phagocytes, the flushing action of cilia and body fluids and the possible influence of antibiotics. One or more of these factors may be important in determining expression of virulence.

In the laboratory, various attempts have been made to control these variables and observe expression in the bacterium. The development of the continuous culture apparatus was one of the most significant steps forward. See, for example, Ellwood and Tempest (1972) for one of the first major studies on the use of a chemostat to demonstrate how the composition of the culture medium influences the chemical structure of the cell wall.

The reader is referred to Birkbeck and Penn (1986) and Brown and Williams (1985) for accounts of our present views on phenotypic expression of virulence determinants, and to Meynell and Gooder (1961) for a historical perspective.

Bacterial exotoxins in virulence

Bacterial pathogenesis requires that the pathogen is able to colonize the human or animal host, to multiply on or in host tissues and to avoid antibacterial defences. These events lead to the establishment of a focus of infection and often culminate in significant damage to the tissues; such damage, which is often mediated by exotoxins, may be localized at the site of infection or generalized at distant sites. Bacterial protein exotoxins are thus important determinants of bacterial virulence. However, the galaxy of putative virulence determinants includes cell-associated factors – considered earlier in this Chapter – such as capsules, fimbrial adhesins, outer membrane proteins and extracellular enzymes and exotoxins.

The targets for toxic action are: (1) mucosal epithelial cells, e.g. epithelial cells of the gastrointestinal tract; (2) subepithelial tissues such as blood vessels, connective tissue and leucocytes (systemic effects are often triggered by toxins that act in this way); and (3) internal organs such as the heart, liver, kidney, central nervous system and neuromuscular junctions.

In classic bacterial toxinoses such as diphtheria, tetanus and botulism, the exotoxin produced by the causative organism is of paramount importance in pathogenesis. More commonly, pathogenesis depends on complex interactions, cell-associated factors and extracellular enzymes combining with exotoxins to produce infection and the clinical signs of disease. Indeed, it is only as a result of recent advances in molecular biology that the roles of individual virulence factors have been determined for certain multifactorial pathogens. Even so, the status of certain exotoxins remains only putative at the present time.

The damaging effect of a toxin may result from direct impairment of an essential metabolic function of the target cell or it may be mediated indirectly through release of mediators such as interferons, interleukins, leukotrienes and prostaglandins. Moreover, toxicity does not always involve death or lysis of the target cell: selective impairment of physiological and metabolic functions can result from subtle non-lethal damage at the site of action.

In terms of mode of action, bacterial toxins fall into two main classes: (1) **cytolytic toxins** that damage cell membranes; and (2) **bipartite (A–B) toxins** that bind to a specific receptor through the B (binding) region and then release the toxic A region which causes cell damage, usually at a site within the target cell.

The purification and molecular characterization of bacterial toxins has advanced rapidly in recent years such that the molecular properties of many important toxins are now well established. Knowledge of the mode of toxin action at the molecular level has also progressed rapidly.

In order to deal with this large topic economically, the properties of a number of toxins produced by bacteria pathogenic for man are summarized in Tables 13.7–13.9 in which toxins are arranged according to the target tissue affected in the disease process. Some general features of toxins acting on different target tissues are dealt with in the accompanying text. The recent reviews of Sandvig and Olsnes (1986), Gill (1988), Freer (1988), Robertson (1988), Scotland 1988 and Arbuthnott (1988a, b) are recommended as comprehensive sources of current information on the toxins listed here. In addition, some key references are cited in Tables 13.7–13.9 for each category of toxins.

Toxins acting on the gastrointestinal tract (Table 13.7)

Several bacterial exotoxins are involved in the pathogenesis of intestinal disease and cause the symptoms of watery diarrhoea, dysentery (bloody diarrhoea) or vomiting. Except when these symptoms result from the consumption of preformed toxin, as in the case of staphylococcal food poisoning, the initiation of disease requires entry of organisms into the intestinal tract and colonization of the intestinal lumen through adherence to mucosal cells, followed by elaboration of toxin.

Diarrhoea results either from direct or indirect stimulation of normal pathways of fluid and ion secretion or from selective impairment of absorption.

Enterotoxins causing diarrhoea without inflammation

Examples of this group of enterotoxins include cholera enterotoxin (CT) and the heat-labile (LT) and heat-stable (ST) toxins elaborated by enterotoxigenic (ETEC) strains of *E. coli*. Once cholera vibrios or ETEC organisms have established themselves in the proximal small intestine through adhesin-mediated attachment, the secreted enterotoxins perturb the normal processes of ion and water absorption secretion across the intestinal epithelium.

This disruption is mediated by the elevation of intracellular levels of the cyclic nucleotides cAMP and cGMP which are synthesized upon stimulation of adenylate and guanylate cyclase respectively; these cyclic nucleotides act as intracellular messengers in the complex sequence of events that lead to net secretion of water and electrolytes in the small intestine, which appears as watery diarrhoea.

CT and LT are very similar in molecular structure and have an identical mode of action. Both are bipartite A–B toxins, consisting of five binding (B) subunits and a single active (A) subunit. The A subunit is readily nicked proteolytically into two fragments (A_1 and A_2). Fragment A_2 is part of the delivery system and is linked non-covalently to the five B subunits, whereas fragment A_1 is enzymically active and is responsible for toxicity. Binding to susceptible target cells occurs through the appropriate receptors (CT receptor = G_{M1} ganglioside; LT receptors = G_{M1} ganglioside and glycoprotein).

Enterotoxic activity results from the intracellular action of fragment A_1, which causes ADP-ribosylation of the α-subunit of the GTP-binding regulatory component of adenylate cyclase. This results in activation of adenylate cyclase and raised levels of cAMP which, in turn, trigger electrolyte secretion as described above.

Information from peptide sequencing of purified toxin protein and DNA sequencing of cloned genes reveals a high degree of homology between CT and LT. However, slight differences do exist between LT from human ETEC pathogens and LT from porcine ETEC strains. Furthermore, whereas LT genes are found on transmissible plasmids, the gene for CT is located chromosomally.

The heat-stable enterotoxin (ST_a and ST_b) of ETEC can be differentiated on the basis of methanol solubility. ST_a toxins, which are produced by human and porcine strains, comprise a set of small methanol-soluble peptides which cause water and electrolyte secretion through activation of guanylate cyclase in intestinal epithelial cells. ST_b, a product of porcine ETEC strains, is methanol insoluble and its mode of action does not involve cyclic nucleotides.

Enterotoxins causing structural damage to intestinal cells

A number of enteric pathogens affect the structural integrity of intestinal epithelial cells in the course of disease leading to local inflammation. This occurs as a result of direct toxin-mediated cytotoxity (e.g. *Cl. difficile*, *Cl. perfringens* type A and *B. cereus*), from intimate contact between the pathogen and brush border membranes (e.g. enteropathogenic *E. coli* (EPEC)) or from cell damage following invasion of intestinal epithelial cells and intracellular multiplication (e.g. *Shigella* and enteroinvasive strains of *E. coli* (EIEC). In many cases the determinants of adherence, penetration and multiplication are as important as exotoxin production in the pathogenesis of disease. It is interesting that EPEC, EIEC and enterohaemorrhagic *E. coli* (EHEC) strains produce bipartite toxins known as shiga-like toxins (SLTs) that are immunologically related but not identical to the shiga toxin of *Shigella dysenteriae*.

Toxins causing subepithelial or systemic damage (Table 13.8)

Establishment of a focus of infection in subepithelial tissue by so-called extracellular bacterial pathogens (i.e. pathogens that do not normally invade host cells) requires that the pathogen can resist the barrage of defences at the disposal of the host. There are several points at which

Table 13.7 Toxins that affect the intestine

Pathogen	Disease	Toxin	Molecular structure	Mode of action	Role in pathogenesis
Vibrio cholerae	Cholera	Cholera toxin (CT	Mol. wt 84 000 (A = 28 000, B = 11 500) Subunit structure A:5B	Binds to receptor (G$_{M1}$ ganglioside Enzymatic ADP ribosylation of GTP-binding regulator of adenylate cyclase causes activation of adenylate cyclase	Established
Escherichia coli (enterotoxigenic *E. coli*: ETEC)	Travellers diarrhoea	*E. coli* heat-labile (LT) toxin	Mol. wt 88 000 Subunit structure A:5B as for CT. LT-B subunit shows 80% homology with CT-B	Activation of adenylate cyclase as for CT	Established
		E. coli ST_1 (human type)	Small peptide 17–19 amino acids long 3 disulphide bonds	Activation of particulate guanylate cyclase in intestinal cells following binding to specific receptor	Established
E. coli (enteropathogenic *E. coli*: EPEC)	Infant diarrhoea	Moderate levels of shiga-like toxin (SLT)	At least 2 forms exist: SLT$_1$, SLT$_2$ Shiga-like toxin is an A–B toxin with a subunit structure (A:5B) similar to shiga toxin	Cytotoxic, neurotoxic and enterotoxic like shiga toxin. SLT is immunologically related but not identical to shiga toxin	Established
E. coli (enteroinvasive *E. coli*: EIEC)	Dysentery-like illness	Multiple SLTs			
E. coli (enterohaemorrhagic *E. coli*: EHEC)	Haemorrhagic colitis with copious diarrhoea Haemolytic anaemia syndrome	High level production of SLT			
Shigella dysenteriae	Dysentery	Shiga toxin	Mol. wt 70 000 (A = 32 000, B = 7 700) Subunit structure A:5B	A subunit inhibits protein synthesis by inactivation of 60S ribosomes. Toxin is enterotoxic, cytotoxic and neurotoxic	Established
Clostridium perfringens	Food poisoning with diarrhoea	Cytotoxic enterotoxin	Mol. wt 34 000 single polypeptide chain	Causes cytotoxic damage to intestinal epithelial cells	Established
Cl. difficile	Pseudomembranous colitis	Toxin A	Mol. wt 440 000 Subunits (230 000, 47 000 and 16 000)	Enterotoxic, lethal, causes fluid accumulation, causes haemorrhagic diarrhoea	Established
		Toxin B	Mol. wt 500 000	Lethal, highly cytotoxic for cultured mammalian cells, acts intracellularly	Established
Staphylococcus aureus	Food poisoning with vomiting and diarrhoea	Seven enterotoxins (A, B, C_1, C_2, C_3, D, E)	Toxin A: mol. wt 27 500 Toxin B: mol. wt 28 500 Toxin C_1: mol. wt 27 500 Toxin C_2: mol. wt 26 900 Toxin C_3: mol. wt 27 100 Toxin D: mol. wt 27 200 Toxin E: mol. wt 26 100	Exterotoxins cause fluid imbalance in colon but molecular mode of action on intestine unknown. Thought to stimulate vomiting centre through action on vagus nerve. Also causes systemic effects	Established (all)
Bacillus cereus	Food poisoning with vomiting and diarrhoea	Diarrhoeagenic toxin/lethal toxin	Mol. wt c. 50 000	Causes fluid accumulation, increased vascular permeability, necrosis and lethality; mode of action unknown	Putative

References: Finkelstein (1973); Marques *et al.* (1986); Weikel *et al.* (1986); Wreggett (1986); Finkelstein *et al.* (1987); Levine (1987); O'Brien and Holmes (1987).

the extracellular products of such a pathogen can modify the host response: (1) by inhibiting the inflammatory response at an early stage; (2) by inhibiting the chemotactic response of phagocytic cells; (3) by killing or impairing the function of phagocytic cells that arrive at the focus of infection; (4) by exerting toxic effects within phagocytes after the pathogen has been phagocytosed; and (5) by indirect toxic effects resulting from the action of bacterial products on mediator cells. Once a focus of infection is established, local tissue damage to connective tissue cells, small blood vessels and muscle tissue may result, leading to, for example, abscesses, wound infections, necrosis and gangrene. The pathogens responsible for such lesions (*S. aureus*, *Str. pyogenes* and *Cl. perfringens*) are well endowed with an array of cytolytic membrane-damaging toxins which are candidate determinants in such effects.

Typical membrane-damaging toxins such as staphylococcal α- and β-toxin, streptolysin O and streptolysin S, *Cl. perfringens* α- and θ-toxins all inhibit leucocyte chemotaxis at subcytolytic concentrations; it is of interest that some toxins are more active against polymorphs, and others against macrophages.

At higher concentrations the same group of cytolytic toxins are involved in necrosis and tissue damage. For instance, type A strains of *Cl. perfringens* are the most common cause of gas gangrene, and this disease is an example of a situation in which tissue damage probably

		Streptolysin S	Small polypeptide, mol. wt 1800 that is active only when bound to carrier molecules such as serum albumin, lipoprotein and RNA	A lethal cytolysin active on a wide range of cell types, including human leucocytes	Putative
	Scarlet fever	Erythrogenic toxins (A, B and C)	A, single chain, mol. wt 26 900 B, single chain, mol. wt 17 500 C, single chain, mol. wt 13 200	Toxins are pyrogenic, mitogenic and erythrogenic. Responsible for rash of scarlet fever	Established
Clostridium perfringens	Wound infections, cellulitis and gas gangrene in man. Many toxaemic conditions in animals	α-toxin	Single chain, mol. wt 43 000	Ca^{2+}-dependent phospholipase C that hydrolyses membrane phospholipids and is cytolytic for many cell types. Lethal and necrotizing	Established
	Pig-bel, necrotic enteritis	β-toxin	Single chain, mol. wt 28 000	Lethal, necrotizing and paralytic. Does not act as a general cytolysin. Necrosis of villi in the intestine causes symptoms of necrotic enteritis	Established
		δ-toxin	Single chain, mol. wt 42 000	A lethal cytolytic toxin that affects RBCs, leucocytes and platelets	Putative
		ε-toxin	Single chain, mol. wt 35 600 Activated by tryptic cleavage of *N*-terminal peptide (14 residues)	A lethal and necrotizing toxin responsible for pulpy kidney disease (overeating disease) in sheep	Established
		ι-toxin	Two component toxin ι_A mol. wt 48 000 ι_B mol. wt 70 000	Lethal and necrotizing toxin causing fatal enterotoxaemia in calves and lambs	Established
		θ-toxin	Single chain O-labile toxin. Mol. wt 60 000	Potent cytolysin active against a wide range of cell types. Polymerization of the toxin on cholesterol-containing membranes leads to lysis	
Bacillus anthracis	Anthrax	Anthrax toxin	Multicomponent toxin I, oedema factor (EF); mol. wt 89 000 II, protective antigen (PA), mol. wt 85 000 III, lethal factor (LF); mol. wt 83 000	PA binds to receptor on target cell. Proteolytic nicking of PA creates receptor for either ET or LF with formation of toxic complexes which are internalized EF is an active adenylate cyclase which, when internalized, causes an increase in cAMP Mode of action of LF not understood	Established
Bordetella pertussis	Whooping cough	Pertussis toxin	Mol. wt 105 700 A:B bipartite toxin Subunit structure: $A:S_1 \begin{matrix} \diagup S_2-S_4 \\ \diagdown S_3-S_4 \end{matrix}$ S_2-S_4 and S_3-S_4 dimers act as B region	Binding occurs through B region, allows entry of A subunit. This causes ADP ribosylation of an inhibitory membrane-bound GTP-binding protein, with activation of adenylate cyclase Systemic effects include fever, lymphocytosis, leucocytosis, and impaired regulation of blood glucose	Established

References: Alouf (1986), Freer (1986), Freer and Arbuthnott (1986), Leppla *et al.* (1986), McDonel (1986), Wardlaw and Parton (1986), Arbuthnott (1988).

Table 13.8 Toxins that cause subepithelial or systemic damage

Species	Disease	Toxin	Molecular structure	Mode of action	pathogenesis
Staphylococcus aureus	Wide range of infections, including wound infections, septicaemia, boils, and other primary and metastatic pyogenic lesions	α-toxin	Single chain, mol. wt 33 000	Forms transmembrane channels consisting of hexameric ring structures Cytotoxic damage can result in death, necrosis, paralysis, or neurotoxicity depending on dose and target tissue	Established
		β-toxin	Single chain, mol. wt 30 000	Phospholipase with specificity for sphingomyelin Cytolytic for RBC and for macrophages and platelets	Putative
		γ-toxin	A, two-component toxin γ_1-single chain, mol. wt 32 000 γ_2-single chain, mol. wt 36 000	The mechanism of cytolysis not known. It lyses RBC and certain other cell types	Putative
		δ-toxin	Small amphiphilic peptide, mol. wt 3000	Causes detergent-like lysis of many cell types	Putative
		Leucocidin	A, two-component toxin F, single chain, mol. wt 32 000 S, single chain, mol. wt 31 000	Binding of S followed by F leads to activation of endogenous phospholipase 2, initiation of arachidonic cascade and subsequent cytolysis. Acts on polymorphs and macrophages	Putative
	Scalded skin syndrome and bullous impetigo	Epidermolytic toxins	ET_A, single chain, mol. wt 27 000 ET_B, single chain, mol. wt 27 500	Acts on the stratum granulosum of the epidermis to cause cell separation and subsequent blister formation. Probably disrupts normal cytoskeletal structure through binding and effect on filaggrin	
	Toxic shock syndrome	Toxic shock syndrome toxin 1 (TSST-1)	Single chain, mol. wt 22 000	Induces the symptoms of toxic shock syndrome in animals, i.e. fever, muscle damage, hypotension, lethargy and shock Releases mediators, i.e. TNF and interleukin-1 from macrophages. Molecular mechanism unknown	Established
Streptococcus pyogenes (group A streptococci)	Streptococcal sore throat, wound infection, pyogenic lesions, impetigo Complications: rheumatic fever, glomerulonephritis	Streptolysin O	Single chain, oxygen-labile haemolysin, mol. wt 67 000	One of a group of O-labile cytolysins that polymerize in the form of rings and arcs on susceptible membranes to cause lysis. Haemolytic, lethal, acts on human leucocytes and attacks many other cell types	Putative

Table 13.9 Toxins that attack internal organs

Species	Disease	Toxin	Molecular structure	Mode of action	Role in pathogenesis
Clostridium botulinum	Botulism	Series of neurotoxins A B C D E F G	Toxin A: mol. wt 145 000 Toxin B: mol. wt 155 000 Toxin C: mol. wt 14, 000 Toxin D: mol. wt 170 000 Toxin E: mol. wt 147 000 Toxin F: mol. wt 155 000 All are single chains activated by proteolytic cleavage to yield heavy (B) fragment and light (A) fragment	Toxins act presynaptically at neuromuscular junctions causing flaccid paralysis. They inhibit Ca^{2+}-dependent exocytosis of vesicles containing acetylcholine	Established
		C_2	An A–B toxin containing 2 components C_2 = A component: mol. wt 51 000 C_2 = B component: mol. wt 90 000	C_2 toxin acts as an ADP ribosylating toxin: its role in disease is unknown	Putative
Cl. tetani	Tetanus	Tetanospasmin	Mol. wt 150 000 Single chain activated by proteolytic cleavage to yield a heavy fragment and a light fragment. These probably function as A and B components	Toxin causes spastic paralysis due to inhibition of release of inhibitory neurotransmitter from inhibitory neurons. Heavy component binds to membranes but detailed mechanism still not clear	Established
Corynebacterium diphtheriae	Diphtheria	Diphtheria toxin	Single chain, mol. wt 62 000 Activated by proteolytic nicking and thiol reduction to give 2 fragments A (active) mol. wt 24 000 B (binding) mol. wt 38 000	Binding of B region to receptor followed by nicking leads to entry of A fragment. This causes ADP ribosylation of elongation factor 2 and inhibition of protein synthesis Damages heart, kidney, liver and adrenals	Established

References: Bizzini (1984), Pappenheimer (1984), Habermann and Dreyer (1986), Sakaguchi (1986).

involves several factors. Pathological changes include necrosis and oedema, with damage to connective tissue, blood vessels and muscle tissue; systemic effects can follow rapidly, with the development of a fatal toxaemia. The α-toxin of *Cl. perfringens* has been well characterized and shown to be a phospholipase C. It is a cytolytic, membrane-damaging toxin that exerts its haemolytic, cytotoxic, dermonecrotic and lethal action through degradation of membrane phospholipids. Auxiliary factors implicated along with α-toxin are θ-toxin (a thiol-activated cytolysin), κ-toxin (collagenase) and υ-toxin (hyaluronidase).

Like *Cl. perfringens*, *S. aureus* produces a large number of toxins and auxiliary toxic factors, including a group of membrane-damaging cytolysins (α-, β-, γ-, δ-toxins and leucocidin), each of which is a candidate for involvement in localized tissue damage or more subtle perturbation of host defences. Work with animal model systems, together with the very recent application of molecular cloning techniques involving the creation of site-directed mutants, has established an important role for staphylococcal α-toxin in the initiation of infection. This agent is cytotoxic for a wide range of cells and tissues, and acts by penetrating the hydrophobic interior of mammalian membranes, forming transmembrane pores which destroy the normal permeability properties of the target cell.

The mechanism of cell damage is not always cytolytic. For instance, in whooping cough, the bipartite toxin (pertussis toxin) by its action on adenylate cyclase induces a number of systemic effects including fever, lymphocytosis, leucocytosis, increased sensitivity to histamine and impaired regulation of blood glucose. Such a cascade of effects may, in some cases, result from the action of the toxin on the levels of major regulatory mediators such as interleukins and tumour necrosis factor. This appears to hold for staphylococcal toxic shock syndrome toxin as well as for the staphylococcal enterotoxins and streptococcal erythrogenic toxin.

By contrast, the unusual specificity of staphylococcal epidermolytic toxin for cells in the stratum granulosum of the epidermis strongly suggests that binding to a specific receptor triggers an intracellular signal that initiates the events of cell separation and epidermal splitting, which in turn lead to blister formation or extensive exfoliation of the epidermis typical of the scalded skin syndrome. Even more complex is the systemic damage seen in anthrax, in which the interaction of three distinct proteins is required for lethality.

Toxic damage to internal organs (Table 13.9)

In some diseases, toxin absorbed across an epithelial surface or diffusing out from a focus of infection penetrates through the blood stream and lymphatics or via neurons to affect single or multiple targets in internal organs at sites distant from the focus of infection. Examples of this sort of damage include the classic toxinoses diphtheria, tetanus and botulism.

In classic diphtheria, local invasion of the nasopharyngeal tissues by toxinogenic strains of *C. diphtheriae* leads to the formation of the pseudomembrane of diphtheria, a tough fibrinous sheet containing fibrin, necrotic epithelial tissue, lymphocytes, leucocytes, erythrocytes and very large numbers of diphtheria bacilli. Underlying the pseudomembrane is a necrotic haemorrhagic lesion. In addition to this local lesion, which itself can be fatal due to occlusion of the airways, damage to the internal organs such as the heart, lungs, liver, kidney and nervous system results from uptake and systemic spread of diphtheria toxin. The diphtheria bacilli themselves remain at the focus of infection and are not present in the haemorrhagic, necrotic lesions found in internal organs. As would be expected in such a toxinosis, the presence of circulating antitoxin is protective and toxoid immunization, now practised in all developed countries, has been spectacularly successful in reducing the incidence of the disease. The pathogenesis of tetanus is also characteristic of a toxinosis, and again antitoxic immunity confers protection. Here the site of infection is a wound, where sporing tetanus bacilli grow in the anaerobic conditions created by tissue trauma. Tetanus toxin released at this site reaches its point of attack in the central nervous system by passing along nerve axons. The pathology of tetanus, namely convulsive spasms of voluntary muscles, results from continual excitation of the motor neurons of the central reflex apparatus in the spinal cord. Tetanus toxin exerts this action by preventing the release of the inhibitory neurotransmitters, glycine and γ-aminobutyric acid, that under normal conditions would prevent overstimulation of the motor neurons.

The third classic toxinosis, botulism, is not an infectious disease. In man it results from consumption of small quantities of preformed botulinum toxin produced by *Cl. botulinum* in contaminated food. Botulinum toxins form a group of serologically distinct neurotoxins. They are secreted as single polypeptide chains (mol. wt c. 150000) which, like tetanus toxin, yield heavy and light chains following proteolytic nicking. Botulinum toxins are the most potent known bacterial toxins with toxicities of 10^7–10^8 lethal doses (LD_{50}) per mg for mice. The toxins prevent release of acetylcholine at peripheral nerve endings such as neuromuscular junctions, thereby causing flaccid paralysis. The precise molecular mechanisms involved in the action of botulinum toxins are still to be clarified. However, it appears that binding of the toxin is followed by a series of events that result in blocking of calcium-dependent fusion of synaptic vesicles with the presynaptic membrane.

Conclusion

It is now clear that bacterial exotoxins can induce direct or indirect tissue damage which may take place on epithelial surfaces, in subepithelial tissues or in internal organs. The effects are often subtle and must be seen in the light of the overall pathogenic process that involves additional virulence factors such as cell surface components and extracellular enzymes. Progress in the molecular analysis of these complex interactions is proceeding apace and will play a vital role in developing new strategies of combating infectious disease.

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